Enzymatic C–H Activation

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A Regioselective Biocatalyst for Alkane Activation under Mild Conditions**

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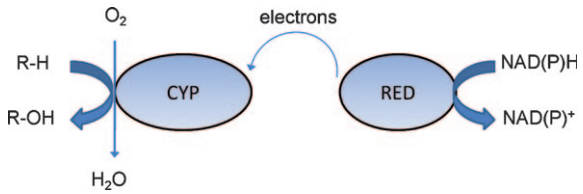
The activation of alkanes to provide compounds of high value is an important scientific challenge.[1] Increasing environmental concerns call for the design of alternative and efficient oxidation routes under mild conditions. Such benign methods should enable the problems associated with the use of toxic acids, solvents, and metals to be circumvented, and should enable reactions to be carried out in an aqueous medium at low temperature with a clean oxidant, such as oxygen.[2] The use of biological catalysts instead of chemical catalysts can be advantageous, because difficult reactions can often be performed under mild conditions. Indeed, alkanes are particularly inert molecules, and their selective transformation remains an important problem.[3]

Cytochromes P450 (CYP) form a superfamily of enzymes widely distributed from prokaryotes to superior eukaryotes.[4] They are known to catalyze the monooxygenation of a large variety of hydrophobic chemical compounds, such as alkanes, xenobiotics, steroids, and prostaglandins.[4c,5] An important element for P450 catalysis is the nicotinamide adenine dinucleotide cofactor, which, in its reduced form (NADH or NADPH), provides the electrons necessary for the catalytic cycle. As the electrons cannot be transferred directly to the heme prosthetic group, redox protein partner(s) (P450 reductase) should be present to shuttle the reducing equivalents from NAD(P)H to the hydroxylase domain[6] (Scheme 1). Slow or uncoupled electron transfer currently limits the catalytic efficiency of P450s.

It is possible to overcome this limitation by artificially fusing a P450 hydroxylase to its natural reductase partner(s)[7] (homologous fusion). Interestingly, functional enzymes can also be obtained by fusion with a reductase from another organism[7] (heterologous fusion). This strategy is of great interest because many P450s catalyze interesting reactions but have no identified reductase. The resulting biosynthetic



Scheme 1. Oxidation of a substrate (RH) under the catalysis of a cytochrome P450 hydroxylase (CYP) coupled to redox protein partner(s) (RED). One molecule of the cofactor (NADPH) and one molecule of molecular oxygen are consumed to yield one molecule of the product (ROH).

single-component cytochromes P450 are self-sufficient: the different partners involved in the reaction remain together, and the coupling efficiency and reaction rate are significantly increased. The cytochrome CYP102A1 from *Bacillus megaterium* (P450 BM3) was the first natural self-sufficient P450 discovered[8,9] and remains one of the most efficient cytochromes to date.

Alkane hydroxylases of the CYP153 family (AH153) are monomeric and soluble proteins that convert medium-chain alkanes (C_5 to C_{12}) into their corresponding alcohols.[10–12] This process is thought to take place in the first step of alkane metabolism, whereby the oxidation of the terminal and/or subterminal carbon atoms leads to 1- and/or 2-alcohols. Such a reaction at room temperature and atmospheric pressure is of great interest to synthetic organic chemists, because the activation of inert C–H bonds remains difficult by classical chemical methods.[2] Pioneering studies on AH153 by van Beilen and co-workers have produced a significant amount of in vitro data for several members of this family.[12] Bioconversions were also conducted by van Beilen et al., who used *Pseudomonas putida* whole cells expressing CYP153A6 from *Mycobacterium* sp. HXN-1500 to produce perillyl alcohol from limonene.[13] In another study, Fujii et al. used *Escherichia coli* to successfully express CYP153A1 from *Acinetobacter* sp. EB104. They produced primary alcohols and α,γ-alkanediols by bioconversion. Although CYP153A1 was correctly folded, functional in vivo, and present in the soluble fraction of cell extracts, its purification and in vitro characterization were not reported.[14] Kubota et al. reported the cloning of large genes encoding several CYP153 hydroxylases fused to an FMN/Fe_2S_2-containing reductase (RhFred, earlier found in the self-sufficient P450RhF from *Rhodococcus* sp. NCIMB 9784[15]) in *Escherichia coli* (FMN = flavin mononucleotide). For experiments with CYP153A13a from *Alcanivorax borkumensis* SK2, the P450 hydroxylase domain was correctly folded, and its in vivo hydroxylation activity enabled the production of primary alcohols by resting cells.[16] How-

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ever, no purification or in vitro analysis was reported for this enzyme, and, as pointed out by Li et al., the self-sufficiency of the system has not yet been determined unambiguously.[17]

The clear indication by all studies reported so far of a lack of a reliable AH153-expression system in *E. coli* prompted us to develop such a system. In this study, we focused on CYP153A13a artificially fused to RhFred and to a histidine affinity tag. We succeeded in the cloning, functional expression in *E. coli*, purification, and complete in vitro characterization (kinetics, substrate binding) of this enzyme and demonstrated its self-sufficiency. We were also able to use this enzyme in whole-cell catalysis with linear and cyclic alkanes.

After cloning, expression in *E. coli*, and purification (see the Supporting Information for experimental details), we recorded the absolute UV/Vis spectrum of the purified enzyme, A13-red, in the absence of a substrate at room temperature (Figure 1). A maximal absorption peak was

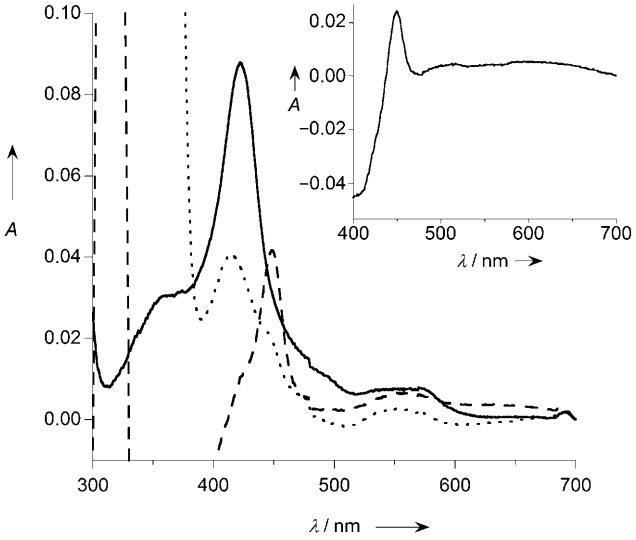

Figure 1. UV/Vis spectra of A13-red. Solid line: oxidized (resting) enzyme; dotted line: dithionite-reduced enzyme; dashed line: dithionite-reduced CO complex. The insert shows the difference spectrum generated by subtraction of the spectra of the dithionite-reduced enzyme and the dithionite-reduced CO complex.

observed at 420 nm. The spectrum showed that 90% of the iron atoms of heme groups were in a low-spin state; thus, the electronic properties of the iron center were not perturbed by the fused reductase. The carbon monoxide difference spectra of the dithionite-reduced protein exhibited the characteristic intense Soret band at 450 nm (Figure 1). This result indicated that the hydroxylase domain of the purified enzyme was correctly folded and functional. Since the hydroxylase and the reductase domains are fused, we assumed that the whole protein was correctly folded. Indeed, the denaturation of part of a protein generally induces denaturation of the entire polypeptide.[18]

The interaction of ferric CYP with a number of substrates involves a concomitant shift in the equilibrium between low-spin and high-spin configurations. Titration experiments were

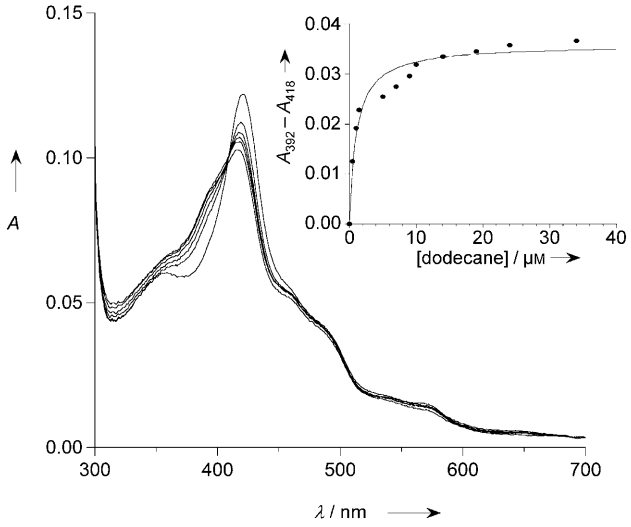

Figure 2. Titration curve of A13-red with dodecane.

performed with the purified enzyme and four different alkanes at room temperature (Figure 2; see also the Supporting Information). Similarly to CYP153A6,[11] but to a lower extent, the chimeric fusion protein strongly interacted with the alkanes (Table 1). The maximal binding affinity was observed with *n*-dodecane ($K_D = 1$ μM), whereas *n*-decane (16 μM) and *n*-octane (29 μM) were found to bind less tightly. Interestingly, cyclohexane induced a significant conversion to the high-spin state, which enabled the dissociation constant to be determined (5 μM). Hence, we showed that hydrophobic molecules, such as linear and cyclic alkanes, have a high affinity for the active site of A13-red, and that its volume is sufficient to enable the docking of cyclic substrates.

We next tested the ability of A13-red to catalyze alkane hydroxylation in vitro, with cyclohexane, octane, decane, and dodecane as substrates. Incubation of the purified fusion protein and the substrate in the presence of NADPH as an electron donor yielded significant amounts of the corresponding alcohols. This result demonstrated unambiguously the self-sufficiency of A13-red. The only product detectable by

Table 1: Dissociation constants, catalytic parameters, coupling efficiencies of A13-red, and bioconversion activities.

Substrate	K_D [μM]	TOF[a] [min^{-1}]	Coupling[b] [%]	TTN[c]	In vivo activity[d]
n-octane	29	57	54	410	60.9
n-decane	16	30	28	235	13.5
n-dodecane	1	0.1	<1	1	2.1
cyclohexane	5	3	25	20	12.7

[a] The turnover frequency was measured by GC/MS analysis over 10 min as (μmol 1-alcohol)/(μmol P450)/min. All reactions were carried out with P450 (150 nM), NADPH (500 μM), and the alkane (4 mM) in potassium phosphate buffer. [b] The coupling efficiency is defined as the ratio between the product-formation rate and the NADPH-oxidation rate. [c] The total turnover number was determined by GC/MS analysis after completion of the reaction as (μmol 1-alcohol)/(μmol P450). [d] In vivo activity is defined as the quantity of 1-alcohol produced in milligrams per gram of cell dry weight (cdw). Values in this table are the mean of at least three independent experiments, and errors were estimated to be 5–10%.

GC/MS after 60 minutes in the reaction with octane at room temperature was 1-octanol. We did not detect any secondary alcohols (such as 2-octanol, 3-octanol, or *i*-octanol) or any overoxidation compounds (such as 1,8-octanediol, octanal, 2-octanone, or octanoic acid). Similarly, the oxidation of decane and dodecane led only to 1-decanol and 1-dodecanol, respectively. These results clearly demonstrate the very high chemo- and regioselectivity of this biocatalyst. High regioselectivity for the terminal hydroxylation of alkanes was also reported for rabbit CYP4B1,[19] for CYP52A3 from *Candida maltosa*,[20] and for AlkB from *Pseudomonas oleovorans*.[21] However, these three enzymes are membrane-bound and require external proteins to be active. Cytochrome CYP153A6 is a very regioselective alkane hydroxylase (>95% ω hydroxylation) but is not a self-sufficient system.[11] Molecular engineering of P450 BM3 has led to artificial alkane hydroxylases; however, even after extensive efforts, hydroxylation occurred mainly at subterminal positions.[22] Alignment of the three-dimensional model of CYP153A6 bound to octane[11,23] with the A13a model (Figure 3a) clearly showed that the 11 residues implicated in the binding of the substrate were 100% conserved and all pointing in the direction of the substrate. This model of A13a provides a good basis to rationalize the affinity of the enzyme for linear alkanes, since it shows that the hydrophobic nature and the volume of these two active sites were highly comparable. Modeling results also showed that the terminal methyl group of linear alkane substrates is very close to (3.2 Å) and ideally positioned above the iron atom of the heme group (Figure 3b). In BM3, it is clear that the ω end of substrates bind in a hydrophobic pocket at one side of the active site distant from the iron center.[24] This arrangement is a key element for explaining the different regioselectivities.

The oxidation of cyclohexane to cyclohexanol with A13-red was also detected during our in vitro assays. This reaction is an important chemical process for the modern chemical industry, because cyclohexanol is a starting molecule for the production of adipic acid and caprolactam, which are intermediates of nylon-6 and nylon-6,6 polymers.[25] This oxidation was more than 99% selective for cyclohexanol (no cyclohexanone or polyol was found).

The purified protein showed a good turnover frequency with octane (TOF = 57 min^{-1}, Table 1). We measured a lower TOF with decane (30 min^{-1}) and cyclohexane (3 min^{-1}). However, almost no activity was observed for dodecane (0.1 min^{-1}) in spite of its high affinity for the enzyme. Therefore, A13-red was preferentially active on octane, although octane did not induce the highest spin change in titration experiments. As observed for wild-type (WT) A6,[11] no direct link between the TOF and K_D values can be made for A13-red. It appears that A13-red is more efficient than the alkane ω-hydroxylases WT A13a (TOF < 0.1 min^{-1} for octane and 8 min^{-1} for decane)[11] and CYP4B1 (two- and threefold less active on octane and decane, respectively).[19] The turnover frequencies were almost the same as those observed for WT A6.[11] Only AlkB surpasses A13-red in terms of TOF (200 min^{-1} with octane[21]); however, as this enzyme was reported to be rather unstable,[27] it can be assumed that the total turnover number (TTN) of AlkB is relatively low.

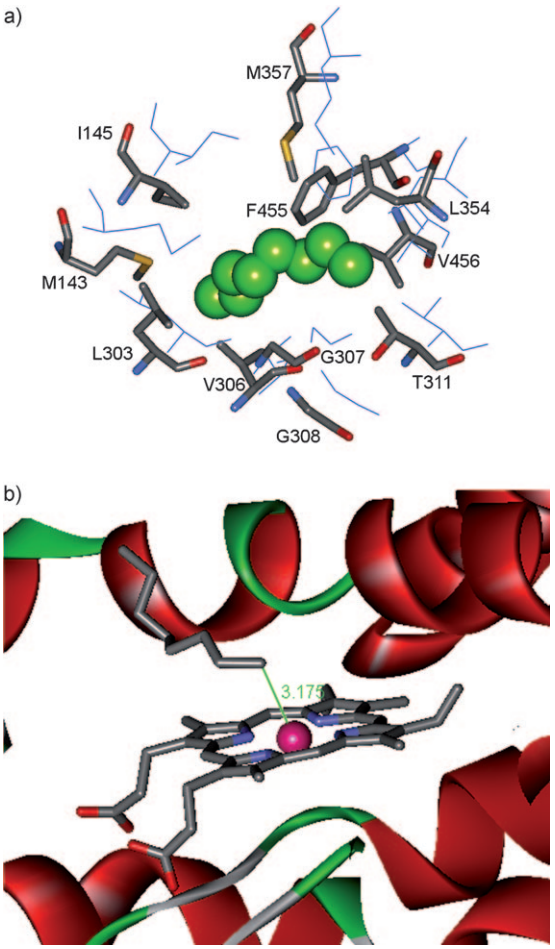

Figure 3. a) Three-dimensional alignment of the CYP153A6 (blue lines) and A13a (gray/colored sticks) active sites docked with octane (CPK model). Only residues in the A13a active site are numbered. b) Zoom-in view of the interaction between the terminal methyl group of octane and the reactive iron center.

Many cytochromes P450 show a significant degree of uncoupling:[28] electrons are consumed from NADPH but do not all participate in substrate oxidation. Coupling efficiency was monitored on the basis of both the NADPH-oxidation and product-formation rates. We observed that, for linear alkanes, there was a linear relationship between coupling efficiency and the TOF value. The hydroxylation of octane was well-coupled to NADPH consumption (54%), whereas with decane it decreased to 28%, and it was less than 1% with dodecane. Different coupling efficiencies for alkane hydroxylases have been described. For example, the double mutant of CYP102A3 from *Bacillus subtilis* (BS1) shows 5% coupling with modest regioselectivity (48% 1-octanol),[29] and the mutant 1-12G of BM3 shows 37% coupling with good regioselectivity (82% 2-octanol).[30] However, no coupling data were reported for WT A6, A13a, or AlkB. Hence, we report for the first time an efficiently coupled alkane ω-hydroxylase.

During uncoupling, reactive oxygen species, such as H_2O_2, are generated. They are responsible for cytochrome P450 inactivation, primarily through degradation of the heme

prosthetic group.[31] For octane hydroxylation, the total turnover number reaches a value of 410, whereas it is lower for decane (235). This lower value might be due to the larger amounts of H_2O_2 produced and is a logical result given the limited tolerance of A13-red for H_2O_2 (see the Supporting Information) and the fact that the TTN reflects the stability of the catalyst during the reaction.

The use of growing or resting whole cells as biocatalysts is an attractive approach when an expensive cofactor, such as NADPH, is required.[32] Resting cells are recovered from an expression culture broth, and bioconversion is performed in a simple aqueous buffered solution containing the substrate (see the Supporting Information). Bioconversions with the same four alkanes were conducted, and the expected 1-alcohols were produced. A maximal productivity of 61 mg 1-octanol per gram of cell dry weight (cdw) was reached for octane, whereas it was lower for decane and dodecane (Table 1). Thus, for linear alkanes, the amount of 1-alcohol produced in vivo correlated with the in vitro TOF value. The productivity of bioconversion with cyclohexane was relatively high in view of the poor in vitro TOF value. One may postulate that cyclohexane enters the cells very rapidly but is also more toxic than decane.[33] Consequently, productivities become comparable despite large differences in vitro. Finally, bioconversion with octane produced 2-octanol (< 5 %), the reaction stopped after 5 hours, and the 1-octanol concentration diminished after this time.[34] Octanoic acid was detected after 24 hours. This product might form by the overoxidation of 1-octanol by enzymes in *E. coli*.[35,36]

In conclusion, our work provides a proof of concept and evidence that CYP153A13a fused to RhFred is a reliable alternative to existing biocatalysts. This self-sufficient, soluble, and efficient enzyme is a step forward towards environmentally benign alkane-activation catalysts that operate in an aqueous medium at low temperature and with oxygen. This biocatalyst shows regio- and chemoselectivities that are much higher than those of any existing enzymes and thus adds to the toolbox available to synthetic organic chemists for alkane oxidation. It may be an excellent starting point for X-ray crystallography studies and directed-evolution experiments devoted to the oxidation of shorter-chain alkanes.

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