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Cytochrome P450 monooxygenase from *Clostridium acetobutylicum*: A new α-fatty acid hydroxylase

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Abstract

Cytochrome P450 monooxygenase from the anaerobic microorganism *Clostridium acetobutylicum* (CYP152A2) has been produced in *Escherichia coli*. CYP152A2 was shown to bind a broad range of saturated and unsaturated fatty acids and corresponding methyl esters and demonstrated a high peroxygenase activity of up to 200 min^{-1} with myristic acid. Although a high concentration of hydrogen peroxide of 200 μM was necessary for high activities of the enzyme, it led to a fast enzyme inactivation within 2–4 min. This might reflect the natural function of CYP152A2 as a rapid hydrogen peroxide scavenging enzyme. In two different reconstituted systems with NADPH, CYP152A2 was able to convert 10 times more substrate, if provided with flavodoxin and flavodoxin reductase from *E. coli* and even 30–40 times more substrate with the CYP102A1-reductase from *Bacillus megaterium*. According to the clear preference for hydroxylation at α-position, CYP152A2 can be referred to as fatty acid α-hydroxylase.

Keywords: *Clostridium acetobutylicum*; P450 monooxygenase; Fatty acids; Peroxygenase; Activity reconstitution

The cytochrome P450s belong to a family of ubiquitous heme *b* containing monooxygenases that play pivotal roles in the detoxification of xenobiotics as well as in the secondary metabolism. P450 monooxygenases catalyze the introduction of one atom from molecular oxygen, with the other reduced to water. The basic P450 catalyzed reactions include hydroxylation of sp3-C atom, heteroatom oxygenation, epoxidation of double bond, and dealkylation (heteroatom release). Two electrons in form of hydrid ion required for P450 catalysis are delivered from NAD(P)H via flavoprotein and/or iron–sulfur redox partners. There are, however, P450 monooxygenases that do not need NAD(P)H and use peroxides for catalysis.

One would expect the presence of P450 monooxygenases exclusively in aerobes, since molecular oxygen is necessary for P450 functions. Nevertheless, the recent genome sequence of the anaerobic microorganism *Clostridium acetobutylicum* revealed a gene encoding for a P450 monooxygenase [1]. *C. acetobutylicum* is an endospore-forming microorganism, which is widely used for solvent production. Although bacteria belonging to the genus *Clostridium* are strictly anaerobic, they can tolerate microoxic conditions (up to 5% oxygen). As reported in the literature, molecular oxygen has a crucial effect on the growth of clostridia. However, the mechanism of growth inhibition and adaptive response to oxygen stress are not completely understood yet. Oxygen detoxification systems in clostridia include superoxide dismutases, superoxide reductases, peroxidases and rubrerythrin, which are responsible for reactive oxygen species (ROS) and intracellular peroxides scavenging [2–4]. Heme oxygenase in *C. tetani* and H_2O-forming NADH oxidase (noxA) in *C. aminovalericum* have

Abbreviations: P450, cytochrome P450 monooxygenase; IPTG, isopropyl-β-D-thiogalacto-pyranoside.

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also been reported as being involved in the establishment of an anoxic microenvironmental conditions [5,6]. Kawasaki et al. [6] reported that many proteins are directly and indirectly involved in oxygen response in *C. acetobutylicum* and suggested that not only oxygen metabolism but also active oxygen and lipid peroxide scavenging enzymes are important for the microoxic growth of this bacterium.

Sequence analysis revealed a 57% identity of the P450 enzyme from *C. acetobutylicum* with CYP152A1 ($P450_{Bs\beta}$) from *Bacillus subtilis* and therefore is considered as its ortholog. $P450_{Bs\beta}$ is a hydrogen peroxide driven fatty acid hydroxylase with mechanism different from most P450 monooxygenases [7,8]. A P450 monooxygenase that consumes molecular oxygen or hydrogen peroxide might also be involved in the oxygen metabolism in *C. acetobutylicum* and therefore is of potential interest from several points of view. Characterization of this enzyme would help to understand better its natural function in *C. acetobutylicum* and probably can give a rise for potential utility as a biocatalyst. Herein, we describe cloning and expression of CYP152A2 (in the following referred to as $P450_{CLA}$) from *C. acetobutylicum* in *Escherichia coli* and its biochemical characterization in terms of stability and substrate spectra.

Materials and methods

Chemicals and enzymes. All chemicals were purchased from Fluka (Buchs, Switzerland). Restriction endonucleases, T4 DNA ligase, Pfu DNA polymerase, IPTG, and SDS–PAGE protein ladder were obtained from Fermentas (St. Leon-Rot, Germany). NADPH was from Jülich Fine Chemicals (Jülich, Germany).

Cloning of $P450_{CLA}$ and $P450_{Bsb}$ encoding genes. All DNA manipulations and microbiological experiments were carried out by standard methods [9]. The gene encoding CYP152A2 in *C. acetobutylicum* ATCC 824 (GenBank Accession No. CAC3330) was amplified by PCR and cloned into pUC18 vector using primers 5′-GGGCTGCAGTTGGAAAAATAAAATATTTAATATATAAGAAAGGAGG-3′ and 5′-GGGGTCGACTATGATTAATGTAATGAATGTAAATTAATTCC-3′. Using this plasmid as template the P450 gene was further amplified by PCR using the oligonucleotide primers 5′-GCTAGCTAGCATGTTACTAAAAGAAAATAC-3′ and 5′-CCGCTCGAGTTAAAGCTTTAGATTAATATTATC-3′. Genomic DNA of *B. subtilis* subsp. subtilis str. 168 was isolated using the standard phenol/chloroform precipitation protocol [9] and the *ybd*T-gene encoding CYP152A1 (GenBank Accession No. BSU02100) was amplified by PCR using primers 5′-CGGGATCCATGAATGAGCAGATTCCACATG-3′ and 5′-CCGCTCGAGTTAACTTTTTCGTCTGATTCC-3′. Thermocycle program was as follows: 25 repetitions of 95 °C for 1 min followed by annealing at 53 °C and extension at 72 °C for 3 min. The amplified genes were purified and fully sequenced by automated DNA sequencing (GATC-Biotech, Germany). The PCR products were digested by endonucleases using appropriate restriction sites introduced during PCR (NheI and XhoI for $P450_{CLA}$ or BamHI and XhoI for $P450_{Bs\beta}$, respectively) and then ligated into the pET28a(+) expression vector (Merck Biosciences, Novagen, Darmstadt, Germany).

Overexpression and purification of P450 monooxygenases. The P450 genes were expressed in *E. coli* BL21 (DE3) cells harbouring a pET28a(+) plasmid containing the gene under control of the T7 phage promoter. Therefore, 200 µl of competent *E. coli* cells were transformed with the desired plasmid and the transformation mixture was used directly to inoculate 400 ml Luria-Bertani broth containing 30 µg/ml kanamycin. Cultures were grown overnight at 37 °C. Recombinant protein expression was then induced by addition of 1 mM IPTG (from 1 M stock in water), the incubation temperature was lowered to 30 °C and the culture was grown for 4 h. The cell pellet harvested from the culture by centrifugation was suspended in 6 ml of 50 mM Tris–HCl buffer, pH 7.5 and the cells were lysed by sonication on ice. Cell debris were removed by centrifugation at 20.000*g* for 20 min at 4 °C and the supernatant was recovered. Concentrations of the correctly folded P450 enzymes were estimated through CO differential spectra as described by Omura and Sato [10,11] using $\varepsilon_{450-490} = 91\ mM^{-1}\ cm^{-1}$. UV/vis spectra were recorded at 24 °C on an Ultraspec 3100pro spectrophotometer (Amersham Biosciences, UK).

For purification, the crude protein extract was diluted in purification buffer (50 mM Tris–HCl, pH 7.5, containing 500 mM NaCl and 20 mM imidazol) and loaded on a Ni–NTA Superflow column (50 × 10 mm, Qiagen, Hilden, Germany) equilibrated with two column volumes purification buffer. Unspecifically bound proteins were removed by a washing step with two column volumes of purification buffer, containing 50 mM imidazol. The bound P450 was eluted with purification buffer, containing 200 mM imidazol and dialyzed in 2 l 50 mM Tris–HCl, pH 7.5, in order to remove imidazol.

Cloning and overexpression of CYP102A1-reductase. CYP102A1-reductase was expressed from the pET28a(+) plasmid carrying the part of CYP102A1 gene coding for a diflavin reductase. The gene fragment was amplified by PCR using the following primers: 5′-GCGGATCCATGAAAAAGGCAGAAAACGC-3′ and 5′-CGGAATTCTACCCAGCCCACACGTCTTTTGCG-3′ and cloned into pET28a(+) vector after digestion with BamHI and EcoRI endonucleases. The heterologous expression of the diflavin reductase was done as described for CYP102A1 holoenzyme previously [12].

Overexpression and purification of flavodoxin (Fld) and NADPH-flavodoxin reductase (Fpr). Fld and Fpr from *E. coli* were homologously expressed *E. coli* BL21 (DE3) from the pET11a plasmid containing a corresponding gene. Expression and purification were carried out as described elsewhere [13].

Spin-state shift and substrate binding constant determinations. Spin-state shifts upon substrate binding were assayed at 24 °C under aerobic conditions through substrate titrations by adding small (<5 µl) aliquots of a 5 mM stock of substrate or ligand dissolved in DMSO to a 1.5 µM P450 enzyme solution and recording spectral changes between 350 and 450 nm. An equal amount of DMSO was added into the reference sample for each measurement. The binding constants (K_D) were calculated by fitting the peak-to-through difference in absorbance to a Lineweaver-Burk type diagram using the Origin 7 program (Origin Labs).

Substrate conversion by P450 and product identification. Three different reconstituted enzyme systems were developed to measure substrate conversion by $P450_{CLA}$ and $P450_{Bs\beta}$. The first system utilizes hydrogen peroxide, while the second and third ones are based on the use of oxygen and NADPH. Activity measurements were carried out in two different sets: one set of experiments (setup A) was performed in 50 mM Tris–HCl buffer, pH 7.5, containing 0.15 µM P450, 60 µM substrate and 200 µM H_2O_2 (in the H_2O_2-based system) or alternatively 300 µM NADPH with 0.3 µM of each Fld and Fpr (second system) or 0.3 µM diflavin reductase of CYP102A1 (third system) in a total volume of 200 µl.

The other set of experiments (setup B) was carried out with higher enzyme concentrations, containing 2.5 µM P450 enzyme solution in 50 mM Tris–HCl buffer, pH 7.5, in a total volume of 500 µl. The H_2O_2-based system contained 25 µM substrate and 50 µM H_2O_2, while the NADPH-based systems were set up with 250 µM substrate and 500 µM NADPH and either 5 µM of each Fld and Fpr (second system) or 5 µM diflavin reductase of CYP102A1 (third system). As a negative control, P450-free cell lysate from *E. coli* harbouring an empty pET28a(+) vector was used.

Substrate conversion was carried out at 37 °C for either 1, 2, or 4 min (setup A) or for 60 min (setup B). Conversion was stopped by adding 20 µl 37% HCl and the reaction mixture was extracted twice with 500 µl diethyl ether. Stearic acid in final concentration 25 or 250 µM was added as internal standard. The combined organic phases were evaporated and the residue was dissolved in 35 µl *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorsilane. One microliter of this solution was analyzed by gas–liquid chromatography–mass spectroscopy (GC–MS) on a GC–MS QP2010 instrument (Shimadzu, Kyoto, Japan) equipped with a

flame-ionization detector (FID) using a FS-Supreme-5 column (0.25 mm × 30 m, Chromatographie Service GmbH, Germany) and helium as carrier gas. The column temperature was controlled at 120 °C for 2 min. The temperature was then raised to 235 °C at the rate of 10 °C/min, and then to 300 °C at 30 °C/min.

Results and discussion

Expression of P450 enzymes

The $P450_{CLA}$-gene from *C. acetobutylicum* and the *ybd*T-gene of *B. subtilis* were amplified by PCR and cloned into standard expression vector pET28a(+). The determined nucleotide sequences of the cloned genes were in complete agreement with those in the genome database. $P450_{CLA}$ (CYP152A2) and $P450_{Bs\beta}$ (CYP152A1) were expressed in soluble form in *E. coli* in reasonable yields (>25 mg/l). Both enzymes were isolated and purified on a Ni–NTA column using advantage of a His-Tag (Fig. 1 for $P450_{CLA}$). The two P450 enzymes showed the characteristic 448 nm absorption for the $Fe^{II}(CO)$ complex with no evidence of the inactive P420 form. Spectroscopic characterization of $P450_{CLA}$ revealed spectra typical for a P450 monooxygenase (Fig. 2).

Substrate binding

Classical Type I substrate binding spectrum of P450 monooxygenases is characterized by a shift in the Soret band from 418 nm in the substrate free form to 390 nm for the high spin form when a substrate is bound in close proximity to the heme to displace the water bound to the iron. Therefore, this method was used for determination

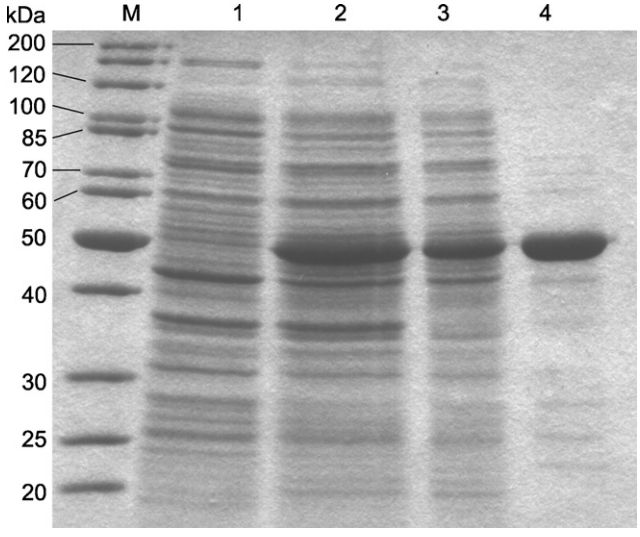

Fig. 1. SDS–polyacrylamide gel electrophoresis at various steps of $P450_{CLA}$ expression and purification. Lane 1, total cell protein before induction; lane 2, total cell protein 4 h after $P450_{CLA}$ expression was induced with 1 mM IPTG; lane 3, crude extract after sonication and centrifugation; lane 4, $P450_{CLA}$ with an estimated size of 48 kDa purified by Ni–NTA chromatography; M, PageRuler™ Unstained Protein Ladder.

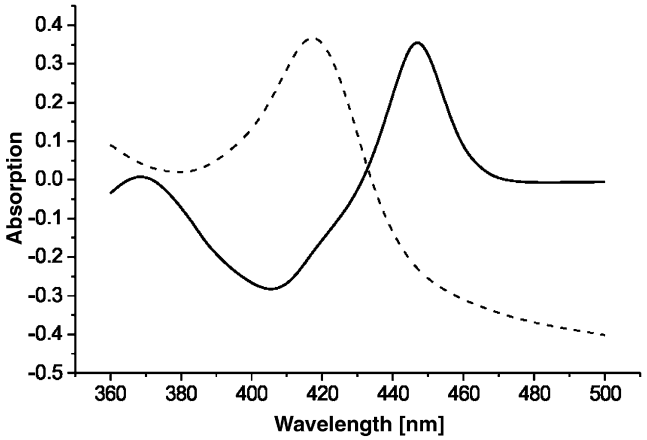

Fig. 2. Spectra of $P450_{CLA}$ in its different redox states: oxidized (dotted line); CO differential spectra (solid line).

of substrate spectra of $P450_{CLA}$. The screening included unsaturated and saturated fatty acids with chain lengths ranging from C_8 to C_{18}, several fatty acid esters, linear alkanes (C_8–C_{16}), and heteroaromatic and polycyclic aromatic compounds (Fig. 3).

No spin-state shift was induced in both P450 enzymes when chosen heteroaromatic and polycyclic aromatic compounds or alkanes were added to the enzymes, although $P450_{Bs\beta}$ has recently been reported to accept anthracene, 9-methyl-anthracene and azulene [14].

$P450_{CLA}$ showed Type I shift with a variety of fatty acids (data not shown). The lowest K_D values were calculated for myristic, pentadecanoic and hexadecanoic acid. Fatty acids with shorter or longer chain length tend to weaken the binding, resulting in increasing K_D values (Table 1). In general, $P450_{CLA}$ was shown to bind a broad range of saturated and unsaturated fatty acids similar to $P450_{Bs\beta}$. However, spin-state shifts for $P450_{CLA}$ could in general only be observed at higher substrate concentrations meaning that higher K_D values were calculated for this enzyme compared to $P450_{Bs\beta}$ (Table 1).

We were surprised to see that spin-state shift could also be observed when the fatty acid was substituted with a corresponding methyl ester. For $P450_{Bs\beta}$ it was previously reported that Arg242 (which is conserved in the $P450_{CLA}$ sequence as well) is a crucial residue for substrate binding,

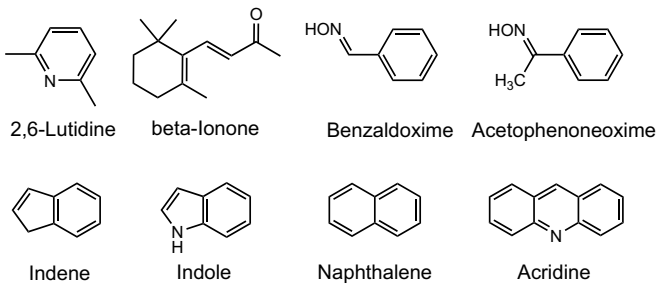

Fig. 3. Heteroaromatic and polycyclic aromatic substrates assayed for $P450_{CLA}$ and $P450_{Bs\beta}$ in this study.

Table 1
K_D values for $P450_{CLA}$ and $P450_{Bs\beta}$ determined from substrate induced Type I spin-state shifts

Substrate	K_D value for $P450_{CLA}$ (μM)	K_D value for $P450_{Bs\beta}$ (μM)
Caprylic acid	>1000	422 ± 27
Capric acid	>1000	414 ± 25
Lauric acid	221 ± 22	88 ± 15
Tridecanoic acid	164 ± 72	15 ± 2
Myristic acid	36 ± 5	9 ± 0.5
Pentadecanoic acid	25 ± 5	8.5 ± 0.5
Palmitic acid	30 ± 6	10 ± 0.5
Heptadecanoic acid	98 ± 21	8.5 ± 1.5
Stearic acid	98 ± 31	40 ± 17
Oleic acid	33 ± 3	22 ± 3
Linolenic acid	70 ± 14	34 ± 4
Arachidonic acid	n.d.	50 ± 5
Stearic acid methylester	690 ± 100	1220 ± 440
Palmitic acid methylester	105 ± 10	72 ± 18
Myristic acid methylester	74 ± 13	30 ± 3
Lauric acid ethylester	(−)	(−)
Lauric acid butylester	(−)	(−)
n-Octane	(−)	(−)
Decane	(−)	(−)
Dodecane	(−)	(−)
Hexadecane	(−)	(−)
Cyclohexene	(−)	(−)
Aromatic compounds (see Fig. 3)	(−)	(−)

n.d., not determined; (−), no spin-state shift observed.

interacting with the negatively charged carboxy group of the fatty acid via hydrophobic interaction and thus determining the regioselectivity of this enzyme. Mutation of this residue by site-directed mutagenesis results in a great inhibition of hydroxylation of myristic acid [15,16]. However, a weakening of this interaction by substitution of the carboxy group with a methylester group still allows the substrate to bind, although with lower affinity compared to the corresponding fatty acid (Table 1). Interestingly, when the carboxy group was substituted with an ethylester or butylester, a spin-state shift could not be observed.

Activity reconstitution and identification of oxidation products by GC–MS

Conversion of lauric, myristic or palmitic acid by $P450_{CLA}$ was investigated with 200 μM H_2O_2. GC–MS analysis demonstrated that $P450_{CLA}$ was able to catalyze the hydroxylation of fatty acids to produce the α- and β-hydroxylated derivatives with quite high initial activities (Table 2). After 2 min of reaction conversion of 60 μM myristic acid reached 40%, lauric acid—55%, and palmitic acid—60%. However, product formation was observed only in the first minutes of the reaction. Presumably, the high concentration of H_2O_2 led to a rapid inactivation of enzyme resulting in a complete loss of activity (data not shown).

To increase stability of the system, we increased enzyme concentration (2.5 μM) and decreased H_2O_2 concentration (50 μM). This resulted, however, in worse productivity: after 1 h only 8% of 25 μM lauric acid, 34% of myristic acid, and 10% of palmitic acid were converted (Table 3). Thus, a high excess of hydrogen peroxide is necessary for high enzyme activity.

To study whether $P450_{CLA}$ accepts oxygen we used two NADPH-based reaction systems to reconstitute the activity of $P450_{CLA}$ and replace the electron donor H_2O_2. There is no evidence for $P450_{CLA}$ or $P450_{Bs\beta}$ belonging to a certain type of P450 in terms of electron partner. Moreover, for $P450_{Bs\beta}$ it is reported that ferredoxin with ferredoxin reductase and cytochrome P450 reductase systems do not appear to function with this enzyme [7]. Nevertheless, we

Table 2
Initial activities[a] of $P450_{CLA}$ and $P450_{Bs\beta}$, measured within 2 min using setup A

Electron donor and substrate	H_2O_2			Fld and Fpr from *E. coli*	CYP102A1-reductase		
	Lauric acid	Myristic acid	Palmitic acid	Myristic acid	Lauric acid	Myristic acid	Palmitic acid
$P450_{CLA}$	108.2	194.8	115.4	(−)	(−)	5.9	1.0
$P450_{Bs\beta}$	38.3	72.2	15.6	(−)	9.9	24.1	14.5

(−), no activity observed.
[a] The activity was calculated in nmol substrate per min per nmol P450.

Table 3
Substrate conversion by $P450_{CLA}$ and activity[a] measured within 60 min using setup B

Electron acceptor and substrate	H_2O_2 ($c_{Substrate}$ = 25 μM)			Fld and Fpr from *E.coli* ($c_{Substrate}$ = 250 μM)			CYP102A1-reductase ($c_{Substrate}$ = 250 μM)		
	Lauric acid	Myristic acid	Palmitic acid	Lauric acid	Myristic acid	Palmitic acid	Lauric acid	Myristic acid	Palmitic acid
Substrate [%][b]	92.1	66.3	90.1	89.6	83.0	88.9	58.2	39.1	69.7
α-Product [%][b]	7.9	22.1	4.7	7.2	11.3	9.8	34.5	51.0	26.4
β-Product [%][b]	0.0	11.6	5.1	3.3	5.7	1.3	7.3	9.9	3.9
Activity[a]	0.8	3.4	1.0	10.4	16.7	11.1	41.8	66.3	30.1

The data are averages of at least three experiments, with standard deviations within ~10% of the mean.
[a] Activity was determined as nmol substrate per h per nmol protein.
[b] %-values correspond to the percental amount of the substance determined by peak integration via GC–MS.

investigated substrate conversion by $P450_{CLA}$ with reconstituted enzyme systems containing either flavodoxin (Fld) and flavodoxin reductase (Fpr) from *E. coli*, which have been described to be structurally similar to the functional domains (FMN binding and NADPH/FAD binding domains, respectively) of NADPH–cytochrome P450 reductases [13] or the diflavin reductase domain of CYP102A1 (P450 BM3) from *Bacillus megaterium*.

Table 4
Substrate conversion by $P450_{Bs\beta}$ and activity[a] measured within 60 min using setup B

Electron acceptor and substrate	H_2O_2 ($c_{Substrate} = 25$ μM)			Fld and Fpr from *E.coli* ($c_{Substrate} = 250$ μM)			CYP102A1-reductase ($c_{Substrate} = 250$ μM)		
	Lauric acid	Myristic acid	Palmitic acid	Lauric acid	Myristic acid	Palmitic acid	Lauric acid	Myristic acid	Palmitic acid
Substrate [%][b]	13.6	1.8	36.1	7.2	5.7	24.8	2.6	2.9	21.5
α-Product [%][b]	12.4	7.0	6.6	31.8	13.8	15.3	34.2	17.4	16.5
β-Product [%][b]	74.0	91.2	57.2	61.0	80.5	59.9	63.2	79.7	62.0
Activity[a]	8.6	9.8	6.4	92.8	94.3	75.2	97.4	97.1	78.5

The data are averages of at least three experiments, with standard deviations within ~10% of the mean.
[a] Activity was determined as nmol substrate per h per nmol protein.
[b] %-values correspond to the percental amount of the substance determined by peak integration via GC–MS.

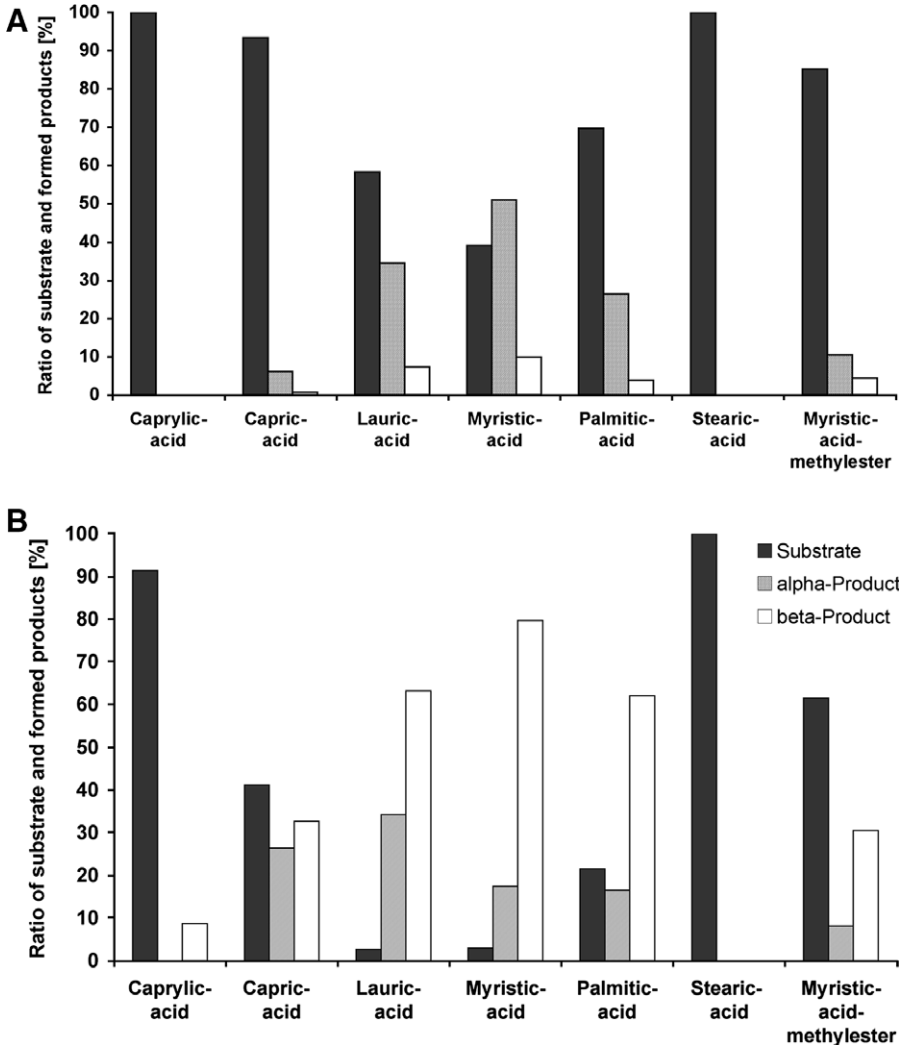

Fig. 4. Hydroxylation of fatty acids by $P450_{CLA}$ (A) or $P450_{Bs\beta}$ (B) using the CYP102A1-reductase reconstituted enzyme system and setup B. After incubation at 37 °C for 1 h, the reaction mixture was analyzed by GC–MS and the ratio of substrate and formed products was determined by peak integration. The data are averages of two experiments, with standard deviations within ~5% of the mean.

The initial activities of $P450_{CLA}$ in both reconstitution systems were either much lower than those measured with H_2O_2 or not measurable at all (Table 2). Nevertheless, since stability of the enzyme was not affected by H_2O_2 in these systems, product formation after one hour was significantly higher than with H_2O_2 (Table 3). $P450_{CLA}$ was able to convert 10 times more substrate, if provided with Fld and Fpr and even 30–40 times more substrate, if CYP102A1-reductase was added to the reaction mixture. Remarkably, the same tendency was observed for $P450_{Bs\beta}$ (Table 4).

For $P450_{CLA}$ the ratio of α- to β-derivatives was ranging from 2:1 to 5:1 (depending on the substrate and conditions chosen). Thus according to the clear preference for hydroxylation at α-position the $P450_{CLA}$ enzyme can be referred to as fatty acid α-hydroxylase.

Using the reconstituted CYP102A1-reductase system we determined the minimal and maximal chain length that is necessary for substrate recognition by both P450 enzymes. Further, we also investigated, if myristic acid methylester can be accepted as substrate. For $P450_{CLA}$ no conversion was detected for caprylic acid (C_8) and stearic acid (C_{18}) and thus even chain fatty acids with chain length between C_{10} and C_{16} are the substrates for $P450_{CLA}$ (Fig. 4A). $P450_{Bs\beta}$ was able to convert small amounts of caprylic acid (Fig. 4B), but was not able to convert stearic acid as well. Both enzymes showed conversion of myristic acid methylester to its α- and β-derivatives. The ratio of α- and β-product was the same as for myristic acid, but after one hour of conversion, 55% or 40% less product was detected for $P450_{CLA}$ or $P450_{Bs\beta}$, respectively.

Conclusions

According to its protein sequence, $P450_{CLA}$ is an ortholog of the peroxygenase $P450_{Bs\beta}$. Indeed, $P450_{CLA}$ demonstrated high activity against fatty acids in the presence of hydrogen peroxide. However, this activity could only be observed within the first few minutes of the reaction. This can be explained by the natural function of $P450_{CLA}$ as an enzyme being involved in hydrogen peroxide scavenging in *C. acetobutylicum*. On the other hand, its role as a member of the oxygen detoxification system can be more complicated, as the enzyme accepts molecular oxygen as well. In this case it would need redox partner(s), providing electrons for catalysis. A flavodoxin is a likely candidate for a physiological electron donor and its identification would provide important information for understanding the role of the P450 enzyme in the anaerobic microorganism.

Acknowledgments

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