Hydroxylation of Fatty Acids*

**Cloning, Expression in Yeast, and Functional Characterization of CYP81B1, a Plant Cytochrome P450 That Catalyzes In-chain Hydroxylation of Fatty Acids**

(Received for publication, November 17, 1997, and in revised form, January 7, 1998)

Francisco Cabello-Hurtado‡§, Yannick Batard‡¶, Jean-Pierre Salaün, Francis Durst, Franck Pinot, and Danièle Werck-Reichhart†

From the Département d’Enzymologie Cellulaire et Moléculaire, Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique UPR 406, 28 rue Goethe, 67000 Strasbourg, France

Several ω and in-chain fatty acid hydroxylases have been characterized in higher plants. In microsomes from *Helianthus tuberosus* tuber the ω-2, ω-3, and ω-4 hydroxylation of lauric acid is catalyzed by one or a few closely related aminopyrine- and MnCl₂-inducible cytochrome P450(s). To isolate the cDNA and determine the sequences of the(ese) enzyme(s), we used antibodies directed against a P450-enriched fraction purified from Mn²⁺-induced tissues. Screening of a cDNA expression library from aminopyrine-treated tubers led to the identification of a cDNA *(CYP81B1)* corresponding to a transcript induced by aminopyrine. CYP81B1 was expressed in yeast. A systematic exploration of its function revealed that it specifically catalyzes the hydroxylation of medium chain saturated fatty acids, capric (C10:0), lauric (C12:0), and myristic (C14:0) acids. The same metabolites were obtained with transgenic yeast and plant microsomes, a mixture of ω-1 to ω-5 monohydroxylated products. The three fatty acids were metabolized with high and similar efficiencies, the major position of attack depending on chain length. When lauric acid was the substrate, turnover was 30.7 ± 1.4 min⁻¹ and *Kₘ* (app) 788 ± 400 nM. No metabolism of long chain fatty acids, aromatic molecules, or herbicides was detected. This new fatty acid hydroxylase is typical from higher plants and differs from those already isolated from other living organisms.

Engineering of lipid metabolism in oilseed crops has become one of the major objectives of plant biotechnology (1–4). Manipulation of fatty acid biosynthesis in transgenic plants offers a possibility for the improvement of the nutritional quality of vegetable oils, but also for redirecting plant metabolism toward production of renewable chemical feedstocks for industrial applications and replacement of petroleum-derived products. Alterations in fatty acid chain length, number, and position of double bonds have already been achieved. Next, valuable modifications will be achieved via introduction of functional groups to confer additional industrial commodities, such as increased or decreased solubility or fluidity, improved solvation of drugs or pesticides, presence of targets for chemical modifications, or synthesis of polymers. Among potential high value metabolites are the oxygenated (hydroxylated and epoxidized) products (5, 6).

Plants are able to synthesize a whole range of oxygenated fatty acids (7). Some of them are major seed storage lipids in particular species. For example, castor bean endosperm produces a seed oil containing up to 90% ricinoleic acid (12R-hydroxyoctadec-cis-9-enoic acid). Other hydroxylated and epoxidized fatty acids constitute the building units of cutin and suberin, biopolymers that form the outer protective layers of all plant species (8). Different types of oxygenated derivatives are also released after mechanical stress or pathogen attack. Some of them play an important role in the signaling pathways and act as triggers of plant defense and plant development (9, 10). Others have been reported to have direct antimicrobial properties and to act as phytoalexins (11–14). Several classes of enzymes are involved in the formation of oxygenated fatty acid metabolites, lipoxygenases, epoxygenases, and desaturases, but the most versatile is the P450 superfamily of monooxygenases. Data obtained with plant microsomes indicate that several P450s catalyze fatty acid hydroxylation or epoxidation with high regio- and stereospecificities (15). Attack of medium chain fatty acids, capric (C10:0), lauric (C12:0), and myristic acids (C14:0), by P450 enzymes occurs at different positions, depending on plant species. Pea and other Leguminosae produce almost exclusively ω-hydroxylated compounds (16, 17). Such ω-hydroxylases are strongly and specifically induced by clofibrate and other peroxisome proliferation-inducing agents (18). In other plant species, attack of medium fatty acids occurs in the chain. Microsomes from grass crops such as wheat and rice generate principally ω-1 and ω-2 hydroxylated metabolites (19). Lauric acid hydroxylation in wheat is induced in an additive manner by phenobarbital and the herbicide safener naphthalic anhydride, an inducer of detoxifying enzymes used for selective protection of crops against herbicide damage (20). A second type of in-chain metabolism was first described in *Helianthus tuberosus* tuber (21–23), but is also observed in several monocots like maize, tulip, or lily (17). It results in the formation of ω-2, ω-3, and ω-4 monohydroxylated products. This type of fatty acid metabolism is strongly induced in response to MnCl₂, aminopyrine, and phenobarbital treatments of plant tissues (17, 22, 24). Parallel induction and inhibition suggest that a single P450 catalyzes the ω-2, ω-3, and ω-4 hydroxylations. High regioselectivity of attack of unsaturated analogues, on the other hand, would rather be indicative of the...
involvement of several and probably closely related enzymes (23, 25, 26).

A new strategy, based on the inducibility of such fatty acid in-chain fatty acid hydroxylases by multiple xenobiotics, has been devised to isolate their coding sequence(s) and to answer the question of their substrate and regiospecificities. We partially purified P450 proteins from MnCl2-induced H. tuberosus tuber tissues (27). Antibodies were raised against the partially purified protein fraction and used to screen a cDNA library from aminopyrine-treated tuber. This study reports the isolation of a cDNA corresponding to aminopyrine-induced transcript coding for a new P450 and the functional characterization of this P450 after expression in yeast. The protein is shown to catalyze the in-chain monohydroxylations of medium C10:0, C12:0, and C14:0 saturated fatty acids. A single P450 accounts for the formation of the different products found in plant microsomes. This new type of fatty acid hydroxylase has never been isolated from other living organisms.

EXPERIMENTAL PROCEDURES

Chemicals—Aminopyrine was from Merck (Darmstadt, Germany). Benzoic acid, salicylic acid, coumarin, and 7-ethoxycoumarin were from Sigma (St. Quentin-Fallavier, France). Other coumarin derivatives were gifts from Dr. J. L. Riviere (Unité de Toxicologie Métabolique et Ecotoxicology, Ecole Vétérinaire, Lyon, France). Phenoxazone derivatives were purchased from Pierce.

3-trans-14C]Cinnamic acid was from Isotop Chim (Ganagobie, France). [1-14C]Iauric acid from CIEF (Gif-sur-Yvette, France), and [1-14C]capric acid from Sigma (La Verpillière, France). [7-14C]Benzoic acid, [1-14C]myristic acid, [1-14C]palmitic acid, [1-14C]oleic acid, [1-14C]linoleic acid were from DuPont (NEN, United Kingdom). [14C]Geraniol and S-[14C]naringenin were kindly provided by Dr. D. Hallahan (IACR-Rothamsted, Harpenden, UK) and Dr. G. Kochs (Institut für Biologie II, University of Freiburg, Germany), respectively. [3-chlorophenyl-14C]Dichlorophenylalanine was kindly provided by Hoechst (Frankfurt am Main, Germany). [triazine-2-14C]Chlorsulfuron by Du Pont de Nemours (Wilmington, Delaware), and [phenyl-14C]Benzon by BASF (Ludwigshaffen, FRG). [phenyl-14C-U]Chlorotoluuron, [phenyl-14C-U]Isoproturon, and [triazine-14C-U]Simazine were generous gifts from Novartis (Basel, Switzerland). [phenyl-14C-U]-2,4-Dichlorophenoxyacetic acid was purchased from Sigma.

Plant Material—Jerusalem artichoke (H. tuberosus L. var. Blanc commun) tubers were grown in open field, harvested in November, and stored in plastic bags at 4 °C in the dark. For aging experiments, tubers were sliced (1.5 mm thick), washed, and aged for 48 h in aerated (4 l/min-1) distilled water containing 20 mM aminopyrine, 8 mM sodium phenobarbital or 25 mM MnCl2. The MnCl2 solution was adjusted to pH 7.

RNA Isolation and RNA Blot Hybridization—Total RNA were isolated as described by Lesot et al. (28). Denatured RNA was separated in the presence of formaldehyde through a 1.2% agarose gel and transferred onto a nylon membrane Hybond N⁺, Amersham Pharmacia Biotech. RNA blots prehybridization and hybridization with a probe, radiolabeled with 50 Ci of [1-14C]oleic acid, and [1-14C]linoleic acid from DuPont (NEN, United Kingdom). [14C]Geraniol and S-[14C]naringenin were kindly provided by Dr. D. Hallahan (IACR-Rothamsted, Harpenden, UK) and Dr. G. Kochs (Institut für Biologie II, University of Freiburg, Germany), respectively. [3-chlorophenyl-14C]Dichlorophenylalanine was kindly provided by Hoechst (Frankfurt am Main, Germany). [triazine-2-14C]Chlorsulfuron by Du Pont de Nemours (Wilmington, Delaware), and [phenyl-14C]Benzon by BASF (Ludwigshaffen, FRG). [phenyl-14C-U]Chlorotoluuron, [phenyl-14C-U]Isoproturon, and [triazine-14C-U]Simazine were generous gifts from Novartis (Basel, Switzerland). [phenyl-14C-U]-2,4-Dichlorophenoxyacetic acid was purchased from Sigma.

Yeast Expression—BamHI and EcoRI sites were introduced by PCR just upstream of the ATG and downstream of the stop codon of the full-length coding sequences, respectively, using the primers 5’TAT-ATAGGATCCATGGATCCCATATCTACTCACC (sense) and 5’TAT-ATAGAATTCGACTCACAGTTCGGACAGTAGATTCGTCATC (reverse). The PCR mixture was preheated for 2 min at 92 °C before addition of 1 unit of Pfu DNA polymerase (Stratagene). After 3 min of additional heating at 92 °C, 30 cycles of amplification were carried out as follows: 1-min denaturation at 92 °C, 1-min annealing at 52 °C, and 2-min extension at 72 °C. The reaction was completed by 10-min extension at 72 °C.

Analytical Methods and Enzyme Assays—Fatty acid metabolism was assayed as in Pinot et al. (33). Metabolites were resolved by thin layer chromatography (Silica Gel G60 F254, 0.25 mm, Merck) with a mixture

![FIG. 1. RNA blot analysis of CYP81B1 expression in H. tuberosus tuber tissues wounded and treated with xenobiotics.](http://www.jbc.org/content/275/7/7261.full.html)
of diethylether/light petroleum (boiling point, 40–60 °C)/formic acid (70:30:1, v/v/v) for C10 to C14 fatty acids, or a mixture of the same solvents (50:50:1) for long chain fatty acids. The areas corresponding to polar metabolites generated from each substrate were scrapped directly into counting vials or eluted with diethylether and subjected to reverse phase-HPLC analysis using a mixture of water/acetonitrile/acetic acid (25:75:0.2) and (45:55:0.2) as described previously (23, 33). Radioactivity of reverse phase-HPLC effluents was monitored with a computerized on-line solid scintillation counter (Ramona-D RAYTEST, Germany). The fluorimetric assays for 7-alkoxycoumarins and 7-alkoxyresorufins O-dealkylation were described in Werck-Reichhart et al. (34). Tests of diclofop, 2,4-dichlorophenoxyacetic acid, chlorsulfuron, and chlorotoluron metabolism were carried out as described by Zimmerlin et al. (19). Tests of isoproturon and simazine metabolism were as for chlorotoluron, except TLC solvents were hexane/chloroform/acetone/ethanol 8:8:4:1 (v/v/v/v) and dichlorometane/methanol/formic acid 90:10:3 (v/v/v), respectively. Assay of bentazon ring-hydroxylation was described in McFadden et al. (35), trans-cinnamate 4-hydroxylation in Reichhart et al. (36), and benzoate 2-hydroxylation in Pierrel et al. (32). Geraniol and abscisic acid hydroxylation were tested according to Vetter et al. (37) and Gillard and Walton (38), respectively.

Spectrophotometric measurements of P450 content were performed as in Gabriac et al. (39). Protein was quantified using the Bio-Rad protein assay. The data are means ± S.D. of triplicate determinations.

Sequence Analysis and Comparison—Double-stranded pBluescript subclones were sequenced using the prism Ready Reaction dye terminator cycle method of Applied Biosystems Inc. The sequence data were analyzed using the BioEdit sequence analysis software package, version 8.1. (40). Sequences were aligned using ClustalW (41) to avoid insertion of gaps in hydrophobic helices. The alignment refined by hand and displayed using SeqVu 1.0.1. (Garvan Institute, Sydney). The tree was drawn using Treeview (42).

Gas Chromatography-Mass Spectrometry Analysis—The oxygenated metabolites of fatty acids were purified by TLC, methylated with diazomethane, and silylated with a mixture of bis-(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane and pyridine (1:1; v/v), before gas chromatography and electron impact (70 eV) ionization mass spectrometry. The analysis was monitored on a 1% SE30 capillary column (30 m x 0.025 mm) programmed to rise from 100 to 280 °C at 6 °C/min, coupled to a LKB 900S mass spectrometer with an LKB 2130 computer on line.

RESULTS

Isolation of the CYP81B1 cDNA—We previously raised antibodies against a P450-enriched fraction partially purified from MnCl2-induced H. tuberosus tuber tissues (27). These antibodies inhibited lauric acid in-chain hydroxylation by about 30%. They were used to screen a cDNA library prepared from tuber tissues treated for 24 h with aminopyrine to induce fatty acid hydroxylase activity. Fifty-six positive clones were isolated and tested for the presence of a P450 consensus sequence using the PCR technique previously described by Meijer et al. (30). PCR fragments of expected size were obtained from 15 clones, labeled, and hybridized with total RNA prepared from dormant, wounded, or aminopyrine-treated tuber tissues. One of the 15 clones corresponded to a transcript of approximately 1.7 kb,
which was undetectable in dormant tuber, became detectable in wounded tissues, and clearly accumulated following treatment with aminopyrine, MnCl$_2$, or phenobarbital (Fig. 1). Sequencing of its insert showed that it coded for a typical P450 missing about 500 nucleotides encoding the N terminus. Re-screening of the library with this insert as a probe led to obtaining the 1DN cDNA still lacking a start codon (Fig. 2), but missing less than 50 nucleotides according to alignment with sequences available in data bases. When submitted to the P450 nomenclature committee (c/o Dr. D. R. Nelson, University of Tennessee, Memphis), the polypeptide encoded by 1DN was assigned to a new P450 subfamily and named CYP81B1.

Obtention of Full-length Sequences—Unexpectedly, the 5′-RACE experiment performed to obtain the missing nucleotides led to the isolation of two cDNA fragments: “s” (376 bp) and “l” (421 bp), both with an ATG start codon preceded by the same 5′-noncoding sequence. s differed from l by two insertions of 14 and 1 amino acids only present in l, two nonconservative amino acid changes, and two silent nucleotide modifications (Fig. 2). It seems unlikely that such a difference could result from a PCR artifact. The N terminus forms the major membrane anchoring segment of P450 proteins. Different membrane anchors may thus reflect different subcellular localizations of two closely related proteins. Comparison of s and 1DN showed that they were identical in their overlaping sequence, and that 1DN was missing only 5 bp from the full-length coding sequence. Two full-length coding sequences were reconstituted from 1DN and s or l, to obtain CYP81B1s (1518 bp; EMBL accession no. AJ000477) and CYP81B1l (1563 bp; EMBL accession no. AJ000478) coding for 505 and 520 amino acids, respectively.

Characteristics of the CYP81B1 Proteins—The CYP81B1s cDNA encodes a protein of predicted $M_r$ 57,060 with a pl of 7.86. In the case of the CYP81B1l construction, the $M_r$ is shifted to 58,877 and the pl to 8.06. In addition to typical $M_r$ and pl, CYP81B1 displays all the signatures of P450 proteins. The PFG(ASV)GRR$^X$C(PAV)G sequence, characteristic of plant P450s from the group A (as defined by Durst and Nelson (43)) and involved in heme binding, is found as expected near the C terminus (Fig. 2). The consensus (AG)$^X$(DE)T(TS), typical of the I helix that participates in oxygen binding and charge transfer in the active site, is also present at the expected location. The N-terminal segment of CYP81B1 is somewhat unusual due to a high content in hydroxylated residues (S and T) and to repetitive alternation of such residues with stretches of hydrophobic amino acids, in particular in CYP81B1l. It can be speculated that the unusual structure of this membrane anchoring segment is related to some particular location of the protein in a specialized cellular compartment or membrane domain. The cluster of basic residues acting as halt-transfer signal and the proline-rich hinge region expected in endoplasmic reticulum bound P450s are present in CYP81B1. That CYP81B1 belongs to the A group of typical plant P450s is confirmed by the comparison of its overall amino acid sequence with proteins of other organisms. CYP81B1 belongs to the same family as CYP81A2 (44% amino acid identity) and show high local identities to the C-terminal region of the parzial CYP81A1, A3, and A4 (46–56% overall identity) isolated from maize (44) (Fig. 3). The other most closely related proteins were P450s isolated from rice and tomato (45).
all belong to the group A of plant P450s, CYP82A1 from pea (40% identity), CYP92A2 from Nicotiana tabacum (38% identity), CYP71A1 from avocado (37% identity), and CYP93A1 from soybean (36% identity). CYP81B1 has only a 34% amino acid identity with the xenobiotic metabolizing CYP76B1 and 29% identity with the cinnamate 4-hydroxylase (CYP73A1) previously isolated from H. tuberosus (45, 46). It shows no phylogenetic relatedness to any of the fatty acid-metabolizing enzymes described so far (Fig. 4).

Optimized Expression in Yeast—Fig. 5 compares the level of expression of CYP81B1s and CYP81B1l in three engineered yeast strains, W(R), WAT11, and WAT21, which overexpress either the endogenous yeast P450 reductase or the Arabidopsis thaliana P450 reductases ATR1 and ATR2, respectively (47). Expression of CYP81B1 and of the different reductases was under the transcriptional control of the artificial galactose-inducible promoter GAL10-CYC1. After 16 h of induction with galactose, no P450 was detected in microsomes prepared from the W(R) strain transformed with either CYP81B1s or CYP81B1l. In contrast, CYP81B1 accumulation was observed in microsomes from WAT11 and WAT21 transformed with either of the two constructions (Fig. 5). CYP81B1 contents were higher in WAT11 than in WAT21: 145 ± 11 versus 118 ± 9 pmol mg⁻¹ protein for CYP81B1s and 40 ± 7 versus 19 ± 5 pmol mg⁻¹ protein for CYP81B1l. Both the length of the membrane anchoring sequence, and the nature of the reductase present in microsomes thus seem to determine the level of CYP81B1 expression in yeast. The most plausible interpretation of these results is that CYP81B1 in yeast microsomes is very labile and that a short membrane-spanning sequence and co-expressed ATR1 both improve the enzyme stability. CYP81B1s recovery from WAT11 was further increased, up to 215 pmol mg⁻¹ protein, by keeping the culture standing at 4 °C for 24 h before preparing the microsomes. The yield in CYP81B1l was, however, not increased by this treatment. Addition of δ-aminolevulinic acid to the induction medium had no effect on the level of expression of CYP81B1.

CYP81B1 Is a Fatty Acid Hydroxylase—Microsomes prepared from WAT11 expressing CYP81B1s or CYP81B1l were used to assay catalytic activity. The molecules tested as substrates are listed in Table I. They include phenolics that are known or potential substrates of P450 enzymes in higher plants. A rapid conversion into polar metabolites was obtained with only capric, lauric, and myristic acids found in higher plants. A rapid conversion into polar metabolites was obtained with only capric, lauric, and myristic acids. The formation of these polar metabolites was dependent on CYP81B1 as shown in Fig. 6 for lauric acid; metabolism of isoprenoids, herbicides, and eight of the most common fatty acids found in higher plants. A rapid conversion into polar metabolites was obtained with only capric, lauric, and myristic acids. The formation of these polar metabolites was dependent on CYP81B1 as shown in Fig. 6 for lauric acid; metabolism required the presence of NADPH and was not detected with membranes of yeast transformed with a void plasmid. All three fatty acids were metabolized with similar and very high efficiencies (Table II). In addition to fatty acid metabolism, a very low chlortoluron ring-methyl hydroxylase activity was detected (kcat ~0.09 min⁻¹ for CYP81B1s in microsomes from transgenic yeast.

**Compared Function and Coupling of CYP81B1s and CYP81B1l**—Previous reports have indicated that changes in the anchoring N-terminal segment of membrane-bound P450s has no critical effect on the catalytic activity and substrate specificity (48–50). This was confirmed by our experiments. The same compounds were metabolized by microsomes prepared from yeast expressing CYP81B1s or CYP81B1l (Table I), although higher specific activities were always measured with CYP81B1s.

The data available so far suggest the existence of multiple

---

I. Benveniste, personal communication.
TABLE I

Molecules assayed as substrates of CYP81B1

| Substrates assayed | Formation of polar metabolites |
|--------------------|--------------------------------|
|                    | CYP81B1s | CYP81B1I |
| Phenolics           |          |          |
| Cinnamic acid       |          |          |
| Benzoic acid        |          |          |
| S-Naringenin        |          |          |
| 7-Methoxycoumarin   |          |          |
| 7-Ethoxycoumarin    |          |          |
| 7-Propoxycoumarin   |          |          |
| 7-Butoxycoumarin    |          |          |
| 7-Methoxresorufin   |          |          |
| 7-Ethoxyresorufin   |          |          |
| 7-Pentoxysorufin    |          |          |
| 7-Butoxysorufin     |          |          |
| Isoprenoids         |          |          |
| Geraniol            | 0.33     | 0.02     |
| Abiesic acid        |          |          |
| Herbicides and drugs|         |          |
| Chlorotoluron       |          |          |
| Isoproturon         |          |          |
| Chlorsulfuron       |          |          |
| 2,4-Dichlorophenoxycetic acid | | |
| Diclofop            |          |          |
| Bentazon            |          |          |
| Simazine            |          |          |
| Aminopyrine         |          |          |
| Fatty acids         |          |          |
| C10:0               | 47.4     | 4.4      |
| C12:0               | 31.5     | 3.5      |
| C14:0               | 14.8     | 2.8      |
| C16:0               |          |          |
| C18:0               |          |          |
| C18:1               |          |          |
| C18:2               |          |          |
| C18:3               |          |          |

P450 reductases in many plant species (43). The specific functions of these multiple reductases has not yet been determined. They may be targeted to different subcellular compartments or membrane subdomains or their differences may just be functional and favor electron transfer to specific P450 proteins. Table III compares the capric acid hydroxylase activity of the s and l constructs in microsomes from yeast strains overexpressing different P450 reductases. No activity was measured in the microsomes from the W(R) strain, which overexpresses yeast reductase, in agreement with the absence of detectable CYP81B1 expression in this strain. The CYP81B1s construct was about 50% more active in the presence of the A. thaliana reductase ATR1 than in the presence of ATR2. By contrast, the specific activity of CYP81B1I was 67% higher in the presence of ATR2 than with ATR1. Different expression of ATR1 and ATR2 reductases does not seem to account for the differences in hydroxylase activity, since NADPH-dependent cytochrome c reduction measured in microsomes from WAT11 and WAT21 was quite similar when the strains were transformed with CYP81B1s (2.92 ± 0.03 and 3.18 ± 0.09 nmol s⁻¹ mg⁻¹ protein, respectively). They were of 5.17 ± 0.14 and 6.83 ± 0.19 nmol s⁻¹ mg⁻¹ for the strains transformed with CYP81B1I. It can be speculated that the different coupling efficiencies of the two CYP81B1 constructs to ATR1 and ATR2 are may be related to the lengths of the membrane anchoring segments of the different proteins, both CYP81B1I and ATR2 sharing longer N termini compared with their CYP81B1s and ATR1 counterparts.

Regiospecificity of the Fatty Acid Hydroxylation—Fig. 6 shows that the same metabolites were produced by microsomes prepared from CYP81B1-transformed yeast and from aminopyrine-treated H. tuberosus tuber. Previous data indicated that several oxygenated derivatives of lauric acid were formed by plant microsomes. They were characterized as the 8-, 9-, and 10-hydroxylated products (21). Gas chromatography-mass spectrometry analysis of the polar metabolites generated from the three fatty acid substrates by CYP81B1 led to the characterization of complex mixtures of monohydroxylated products. In the case of lauric acid, the major methylester trimethylysilyl ether derivatives showed the expected mass fragmentation for hydroxylated fatty acids, with characteristic ions resulting from α,β cleavage of trimethylsilyl ether at m/z 131 and 273 for the 10-hydroxy acid, m/z 145 and 259 for the 9-hydroxy acid, and m/z 159 and 245 for the 8-hydroxy acid. Similarly, fragmentation ions of the major metabolites were characteristic of 9-, 8-, and 7-hydroxylated (m/z 117 and 259, m/z 131 and 245, and m/z 145 and 231) products in the case of capric acid, and of 11-, 10-, and 9-hydroxylated (m/z 145 and 287, m/z 159 and 273, and m/z 173 and 259) fatty acids for myristic acid. The major metabolites were the 9-hydroxy derivatives for capric and lauric acids, and the 10-hydroxylated myristic acid. The best HPLC and gas chromatography resolution of metabolites was obtained for lauric acid. In this case, the estimated ratio of the 10-, 9-, and 8-hydroxylated products was 21/64/15. This is very similar to the ratios measured with plant microsomes and suggests that CYP81B1 is the sole medium chain metabolizing P450 in H. tuberosus tuber tissues.

**DISCUSSION**

A characteristic of P450-dependent fatty acid hydroxylases in higher plants is a very low activity in normal plant tissues (tuber or young shoots). Small increases in activity are observed after mechanical stress, such as wounding or aging of the shoots or tissues, but these increases are negligible compared with the response to some specific chemical treatments (15). This property has been exploited for the isolation of the cDNA coding for H. tuberosus fatty acid-in-chain hydroxylase. Hydroxylase activity is very low in dormant or wounded tuber tissue, but is strongly induced by MnCl₂ or aminopyrine (22, 24). We thus combined use of antibodies directed against partially purified MnCl₂-induced P450s, screening of a library from aminopyrine-treated tissues and selection of clones coding aminopyrine-induced transcripts to isolate the CYP81B1 cDNA. This is the first example of isolation of a plant P450 gene of defined function on the basis of chemically induced expression. Another successful approach taking advantage of differential gene expression has been previously reported (51). It was using a combination of PCR on conserved P450 sequences and selection on the basis of developmental expression, and led to isolation of sequences coding flavonoid hydroxylases involved in the biosynthesis of anthocyanins.

Functional characterization, but also detailed analysis of the substrate-specificity and regioselectivity of CYP81B1 was made possible by the expression of the enzyme in yeast. The yeast expressed protein catalyzes in-chain hydroxylation of C10:0, C12:0, and C14:0 fatty acids. The three substrates are metabolized with high and comparable efficiencies. Shorter fatty acids are also possible substrates; short chain acids being volatile molecules, their metabolism was not assayed. Long chain saturated or unsaturated fatty acids, however, have been assayed. They were not metabolized. Neither were the isoprenoids (geraniol, abiesic acid), aromatic molecules, and herbicides tested in our experiments. CYP81B1 thus seems highly specific for medium chain fatty acids. The high affinity of the enzyme for these molecules (in the 100 nm to 1 μM range) would suggest that they are its natural substrates. From the data obtained with plant microsomes it can be assumed that CYP81B1 is also able to epoxidize or hydroxylate medium chain unsaturated fatty acids (25, 26). Some of the unsaturated acids...
seem to be attacked with a high stereospecificity. Such molecules would be good potential natural substrates of CYP81B1. Nothing is known, however, concerning the possible physiological function of in-chain hydroxylated or epoxidized medium chain fatty acids.

Analysis of the metabolites of capric, lauric, and myristic acids showed that a complex mixture of in-chain monohydroxylated derivatives was generated by CYP81B1. This is probably indicative of some mobility of the molecules in the active site, and could be taken as an argument for the saturated medium chain fatty acids not being the physiological substrates. The major site of attack is at carbon 9 (capric and lauric acid) or 10 (myristic acid) depending on the length of the aliphatic chain. The regioselectivity of the attack thus seems to be primarily determined by the distance relative to the carboxyl end of the molecule. The small influence of the distance to ω-terminus would be easily explained if the ω-terminal methyl was maintained curled away and stuck in a hydrophobic patch of the protein located above heme as in the fatty acid-bound BM-3 structure (52), since the distance of attack to the COOH terminus would increase with the overall bend of the molecule.

Our results demonstrate that a single P450 catalyzes the in-chain hydroxylation of C10:0, C12:0, and C14:0 fatty acids, and that several monohydroxylated positional isomers are generated from the three fatty acids by the same protein. This new P450 is typical from higher plants, and has no known equivalent in other living organisms. One complete and three partial sequences coding for ethanol-induced P450s belonging to the same family (but to another subfamily) have been isolated from etiolated maize shoots (44). The function of these genes has never been determined. It is, however, noteworthy that lauric acid in-chain hydroxylase activity has been detected in maize shoots (17). Two self-sufficient bacterial and fungal cytochromes P450, CYP102 or P450BM-3 from Bacillus megaterium and P450foxy from Fusarium oxysporum, are able to perform in-chain hydroxylation of medium chain fatty acids (53, 54). They differ from CYP81B1 in regiospecificity and substrate specificity. Both enzymes produce ω-1 to ω-3 hydroxylated metabolites. They hydroxylate long chain as well as medium chain fatty acids. In addition, their $K_m$ values for fatty acids are in the 10 to 200 μM range.

In terms of amino acid sequence, CYP81B1 completely dif-

---

**TABLE II**

| Substrate | $K_m$ (nM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$) |
|-----------|------------|------------------------|-------------------------------------|
| C10:0     | 903 ± 168  | 41.0 ± 0.8             | 45                                  |
| C12:0     | 788 ± 400  | 30.7 ± 1.4             | 39                                  |
| C14:0     | —          | —                      | —                                   |

Specific activity of radiolabeled myristic acid was insufficient for accurate determination of the catalytic parameters which were of the same order of magnitude as for capric and lauric acids.

---

**TABLE III**

| Yeast strain | Capric acid hydroxylase activity | CYP81B1s | CYP81B1l |
|--------------|----------------------------------|----------|----------|
| W(R)         | ND                               | ND       | ND       |
| WAT11        | 23.4 ± 0.5                       | 3.9 ± 0.2| 6.5 ± 0.2|
| WAT21        | 15.8 ± 0.7                       |          |          |

ND, not detectable.

---

FIG. 6. TLC analysis of the metabolites formed by microsomes from CYP81B1s-transformed WAT11 yeast and from aminopyrine-induced plant tuber tissues. Microsomes from H. tuberosus (1.2 mg of protein) or from transgenic yeast expressing CYP81B1s (0.1 mg of protein) were incubated 45 min at 27 °C with 100 μM 14C-labeled (top) C10:0, (middle) C12:0, (bottom) C14:0, and 600 μM NADPH. After stopping the reaction with 1 volume of acetonitrile/acetate acid (99:8:0.2), the incubation medium was directly spotted on TLC silica plates. Plates were developed, and radioactivity was recorded as described under “Experimental Procedures.”
fers from the plant, bacterial, fungal, and animal fatty acid oxygenases already characterized. It shares less than 30% identity to other plant P450 enzymes, such as allene oxide synthases and fatty acid hydroperoxide lyases (CYP74s) and the small CYP94 family of fatty acid ω-hydroxylases recently isolated in our laboratory. It also greatly differs from the bacterial CYP102 and from the proteins of the CYP4 family (ω and ω-1 hydroxylases) present in insects and mammals, and from the different hydroxylases and epoxidasases of the mammalian CYP2 family involved either in medium-chain fatty acid ω-1 hydroxylaition or the arachidonic cascade and in the synthesis of signaling molecules (55, 56). The oleate 12-hydroxy-lase, which synthesizes ricinoleic acid in castorseed, is a totally different enzyme both from a mechanistic and phylogenetic point of view and is related to fatty acyl desaturases (57).

CYP81B1 is thus a new and very interesting tool for the modification of fatty acid content of oilseeds and for the orientation of lipid synthesis toward the production of oxygenated derivatives. Redirection of fatty acid synthesis to medium chain acyl-acyl carrier protein thioesterases specific for medium chain acyl-acyl carrier protein isolated from plants accumulating unusual fatty acids such as California bay or Cuphea spp. (3, 58, 59). Combined transformation with CYP81B1 under the control of a seed specific promoter could lead to the production of in-chain hydroxy fatty acids of specific lengths. CYP81B1 has the advantage not to attack the most common C16 and C18 fatty acids, respectively. This P450 is highly specific for fatty acids of a seed specific promoter could lead to the production of 

Acknowledgments—We thank Drs. D. Pompon and P. Urban for providing the W(R), WAT11, and WAT21 yeast strains and the small CYP94 family of fatty acid -hydroxylases recently identified in insects and mammals, and CYP81B1 is thus a new and very interesting tool for the modification of fatty acid content of oilseeds and for the orientation of lipid synthesis toward the production of oxygenated derivatives. Redirection of fatty acid synthesis to medium chain acyl-acyl carrier protein thioesterases specific for medium chain acyl-acyl carrier protein isolated from plants accumulating unusual fatty acids such as California bay or Cuphea spp. (3, 58, 59). Combined transformation with CYP81B1 under the control of a seed specific promoter could lead to the production of in-chain hydroxy fatty acids of specific lengths. CYP81B1 has the advantage not to attack the most common C16 and C18 fatty acids, respectively. This P450 is highly specific for fatty acids of a seed specific promoter could lead to the production of in-chain hydroxy fatty acids of specific lengths.
Cloning, Expression in Yeast, and Functional Characterization of CYP81B1, a Plant Cytochrome P450 That Catalyzes In-chain Hydroxylation of Fatty Acids
Francisco Cabello-Hurtado, Yannick Batard, Jean-Pierre Salaün, Francis Durst, Franck Pinot and Danièle Werck-Reichhart

J. Biol. Chem. 1998, 273:7260-7267.
doi: 10.1074/jbc.273.13.7260

Access the most updated version of this article at http://www.jbc.org/content/273/13/7260

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 56 references, 14 of which can be accessed free at http://www.jbc.org/content/273/13/7260.full.html#ref-list-1