Isolation and Reconstitution of the Heme-Thiolate Protein Obtusifoliol 14α-Demethylase from Sorghum bicolor (L.) Moench*

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The heme-thiolate (cytochrome P450) enzyme which catalyzes the 14α-demethylation of obtusifoliol has been isolated from microsomes prepared from etiolated seedlings of Sorghum bicolor (L.) Moench. The obtusifoliol 14α-demethylase is a key enzyme in plant sterol biosynthesis and a target for the design of phyla-specific sterol 14α-demethylase inhibitors. Microsomal cytochrome P450s were solubilized by using the detergents Renex 690 and reduced Triton X-100, and the obtusifoliol 14α-demethylase was isolated by DEAE ion exchange and dye affinity column chromatography. The isolated enzyme has an absorption spectrum characteristic for low spin cytochrome P450s and produces a Type I binding spectrum with obtusifoliol as substrate. Binding spectra were not obtained with lanosterol, campesterol, sitosterol, or stigmasterol. Obtusifoliol 14α-demethylase has an apparent molecular mass of 53 kDa and is estimated to constitute ~20% of the total cytochrome P450 content of the microsomal membranes and about 0.2% of the total microsomal protein. Gas chromatography-mass spectrometry analysis of reconstitution experiments with dilauroylphosphatidylcholine micelles containing isolated obtusifoliol 14α-oxidoreductase and sorghum NADPH-cytochrome P450 oxidoreductase demonstrated the conversion of obtusifoliol (4α,14α-dimethyl-5α-ergosta-8,24-(28)-dien-3β-ol) to 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol, the 14α-demethylated product of obtusifoliol with a double bond introduced at the Δ14 position. The N-terminal amino acid sequence of the protein is MDLAD-IPQ/KQQRLMAGXALV. Five internal sequences were obtained after endoproteinase Lys-C and Glu-C digestion. The fragment AAGAFSTISFGGGR aligns with the unique heme binding domain of mammalian and yeast sterol 14α-demethylases which belong to the CYP51 family. Therefore it is conceivable that the obtusifoliol 14α-demethylase from plants also belongs to the CYP51 family, the only P450 family so far known to be conserved across the phyla.

Sterol biosynthesis in eukaryotes has diverged into nonphotosynthetic and photosynthetic lineages departing from oxidosqualene (1) (Fig. 1). In the nonphotosynthetic phyla oxidosqualene is cyclized to lanosterol. The 14α-demethylation of lanosterol or its conversion products dihydrolanosterol or eburicol is a key step in the production of ergosterol and cholesterol as the major sterols of yeasts and mammals, respectively (2, 3). The 14α-demethylation reaction is catalyzed by a single multifunctional P450 and proceeds as a sequence of three hydroxylation reactions followed by the elimination of formic acid (4). The 14α-demethylases from yeasts and mammals have been isolated, reconstituted, and cloned (3, 5, 6–13). Sequence and function similarities have placed them in the CYP51 family, which is unique in being the only P450 family to occur in different phyla (14).

Sterol biosynthesis in plants results in the production of sitosterol, stigmasterol, and campesterol as major sterols (15). Numerous biosynthetic studies with radiolabeled putative precursors demonstrated cycloartenol, 24-methylene cycloartenol, cycloeucalenol, and obtusifoliol (Fig. 1, I) as intermediates in the biosynthesis of phytosterols (16–19) but failed to demonstrate the involvement of lanosterol (20). A characteristic feature of the plant pathway is the oxidative C-4 monodemethylation reaction which converts 24-methylene cycloartenol to cycloeucalenol (21). This is in contrast to the nonphotosynthetic pathway where the first demethylation reaction occurs at C-14. The substrate for the 14α-demethylation reaction in plants is thus different from that in animals and fungi. Studies with microsomal membranes isolated from Zea mays (maize) have demonstrated that these catalyze the removal of the 14α-methyl group from obtusifoliol (22). By the inclusion of inhibitors to block additional enzymatic activities present in the microsomal membranes, the demethylation reaction was deduced to result in the simultaneous introduction of a double bond at the Δ14 position. The demethylation reaction was concluded to be catalyzed by a P450 system based on the requirement for NADPH and molecular oxygen as cofactors and the inhibition by carbon monoxide and other typical P450 inhibitors (22). Investigations of the ability of the microsomal system to utilize other substrates for the demethylation reaction (23), the use of t-oxo-obtusifoliol analogs (24) combined with studies on the developmental regulation of sterol biosynthesis (25) indicated that obtusifoliol is the true substrate for the 14α-demethylation reaction in plants.

In the present study we report the isolation and reconstitution of obtusifoliol 14α-demethylase from seedlings of Sorghum bicolor (L.) Moench. The partial amino acid sequence data indicate that the obtusifoliol 14α-demethylase from sorghum, like the 14α-demethylases from mammals and fungi, belong to the CYP51 family, the only P450 family so far known to be conserved across different phyla. All known functional sterols lack the 14α-methyl group (14), and therefore the 14α-demethylation reaction has received much attention from the pharmaceutical and agriculture-chemical industry as a possible means to specifically control and inhibit sterol biosynthesis in mammals, fungi, and plants (26, 27). In this respect, the isolation of obtusifoliol 14α-demethylase from sorghum...
The biosynthesis of sterols in mammals, fungi, and higher plants. The P450-catalyzed obtusifoliol 14α-demethylation step in sterol biosynthesis in higher plants is shown with a bold arrow. The main sterols found in plants are sitosterol, stigmasterol, and campesterol (the α form of 24-methyl cholesterol). In mammals and yeast, the 14α-demethylation occurs on either lanosterol or dihydrolanosterol, yielding cholesterol and ergosterol, respectively, as the main sterol. In filamentous fungi, the 14α-demethylation occurs on eburicol to yield ergosterol. In plants the 14α-demethylation reaction takes place after initial removal of one of the two methyl groups at the C-4 position. (Adapted from Refs. 22 and 36).

EXPERIMENTAL PROCEDURES

Materials—Obtusifoliol isolated from cucumber seeds was a generous gift from Dr. Akhisa, College of Science and Technology, Nihon University, Japan. Obtusifoliol (1 mg) and Tween 80 (47 μl) were dissolved in absolute ethanol (4 ml). The ethanol was removed by lyophilization and the residue containing the sterol, and Tween 80 was dissolved in absolute ethanol (4 ml). The ethanol was removed by lyophilization and the residue containing the sterol, and Tween 80 was dispersed in water to yield a final obtusifoliol concentration of 500 μM and a Tween 80 concentration of 1%. Dispersions of other sterols (Sigma) were prepared accordingly. Renex 690 (Synperonic NP10) was kindly donated by J. Lorentzen AS, Kvistgaard, Denmark. Reduced Triton X-100 was from Aldrich. SDS of Sequanal grade was purchased from Pierce. All other chemicals were of reagent grade or better.

**General Methods**—The elution of P450s and other proteins from the columns was monitored at 420 and 280 nm, respectively, using a Linear UVIS 204 monitor (Linear Instruments Corp.). Total P450 was quantified by difference spectroscopy (SLM AMINCO DW-2C) using an extinction coefficient ε450–490 of 91 mM⁻¹ cm⁻¹ (28) for the adduct between reduced P450 and carbon monoxide. Substrate binding spectra were recorded (SLM AMINCO DW-2000TM) according to Jefcoate (29) by recording the absorbance at 420 nm, by CO binding, and by the presence of proteins in the 50–60-kDa region (SDS-PAGE) were combined (48 ml), diluted 5-fold with buffer C, and applied (flow rate 30 ml/h) to a column (2.8 × 8 cm) of reactive yellow 3A-agarose (Sigma) equilibrated in buffer B augmented with 1% CHAPS (w/v). The column was washed extensively with 150 ml of buffer C, and the P450s that remained bound were eluted with a 0–600 mM KC1 linear gradient (120 ml) in buffer C. The fractions of the eluate that contained P450 as determined by the absorbance at 420 nm, by CO binding, and by the presence of proteins in the 50–60-kDa region (SDS-PAGE) were combined (48 ml), diluted 5-fold with buffer C, and applied (flow rate 30 ml/h) to a column (1.9 × 3.2 cm) of Cibacron blue 3G-A-agarose (Sigma) equilibrated in buffer C.

Preparation of Microsomes and Isolation of Obtusifoliol 14α-Demethylase—All steps involving the preparation of microsomes and the purification of enzyme were carried out at 4 °C. The buffers used for the enzyme purification were degassed by stirring in vacuo and flushed with argon and were prepared as follows: buffer A, 10 mM KPi (pH 7.9), 8.9% glycerol (w/v), 0.2 mM EDTA, 2 mM DTT, 0.05% RTX-100 (v/v), 1% Renex (v/v); buffer B, 40 mM KPi (pH 7.9), 8.9% glycerol (w/v), 5 mM EDTA, 2 mM DTT, 0.05% RTX-100 (v/v), 1% Renex (v/v), 0.1% CHAPS (w/v); buffer C, 40 mM KPi (pH 7.9), 8.9% glycerol (w/v), 5 mM EDTA, 2 mM DTT, 0.05% RTX-100 (v/v), 1% CHAPS (w/v).

P450-containing fractions as identified by their absorption at 420 nm and their CO binding spectra were combined (360 ml), adjusted to 1% CHAPS (w/v), stirred for 20 min, and applied (flow rate 50 ml/h) to a column (2.8 × 8 cm) of reactive yellow 3A-agarose (Sigma) equilibrated in buffer B augmented with 1% CHAPS (w/v). The column was washed extensively with 150 ml of buffer C, and the P450s that remained bound were eluted with a 0–600 mM KC1 linear gradient (120 ml) in buffer C. The fractions of the eluate that contained P450 as determined by the absorption at 420 nm, by CO binding, and by the presence of proteins in the 50–60-kDa region (SDS-PAGE) were combined (48 ml), diluted 5-fold with buffer C, and applied (flow rate 30 ml/h) to a column (1.9 × 3.2 cm) of Cibacron blue 3G-A-agarose (Sigma) equilibrated in buffer C. After washing the column with 40 ml of buffer C, the retained P450s were eluted with a 0–2 x KC1 linear gradient (40 ml) in buffer C. The fractions (10 ml) which by SDS-PAGE showed the presence of a single polypeptide in the 50–60-kDa region were combined, dialyzed for 48 h against 2 × 1 liter of 50 mM KPi (pH 7.9), 5 mM EDTA, 8.9% glycerol (w/v), 2 mM DTT to reduce the salt and detergent content, and concentrated using Centricon 30 concentrators (Amicon) according to the manufacturer. The isolated enzyme was frozen in liquid nitrogen and stored at −80 °C.

Antibody Production, Peptide Generation, and N-terminal Sequencing—The Coomassie Brilliant Blue-stained protein band corresponding to the P450 isolated from the final column was excised from 8–25% SDS-polyacrylamide gels (1 mm × 20 cm × 30 cm) and electroeluted in
a Bio-Rad 422 Electro-Eluter using a buffer composed of 50 mM NH₄HPO₄, 0.1% SDS (w/v) according to the manufacturer, except that 10 mM DTT was included in the upper buffer. The electroelute was cooled on ice, and the precipitated SDS was removed by centrifugation (1 min, 4 °C, 1000 × g).

Antibodies against the electroeluted protein were raised in a rabbit as described previously (32) by injecting approximately 5 μg of protein the first two times and 15–20 μg of protein in the following six immunizations. The antibody fraction obtained from the sixth bleed was used for immunoblotting. The intensity of the bands on the immunoblot were assessed using a Sharp JX-330 scanner (ImageMaster software, Pharmacia). The concentration of the electroeluted protein was estimated by SDS-PAGE.

Electroeluted protein was digested with either endoproteinase Lys-C (18 h, 23 °C) or endoproteinase Glu-C (protease V8, 18 h, 35 °C) of sequencing grade according to the manufacturer (Boehringer Mannheim). The amount of proteinase used was approximately 1/100 of the electroeluted protein by weight.

The electroeluted protein and the fragments generated by protease digestion were subjected to SDS-PAGE at 4 °C using an upper buffer composed of 100 mM Tris, 100 mM Tricine, and 0.1% SDS (w/v) and 200 mM Tris-HCl (pH 8.9) as the lower buffer. Pre-electrophoresis (30 min, 50 V) was carried out in upper buffer augmented with 20 mM thioglycolate, and samples were electrophoresed for 75 min in upper buffer augmented with 1 mM thioglycolate. Protein and protein fragments were transferred to ProBlott membranes ( Applied Biosystems) according to the manufacturer in a semidy blotting apparatus using the Tris-Tricine upper buffer augmented with 0.5 mM DTT as transfer buffer. The ProBlott membrane was stained for 1 min in 0.1% Coomassie Brilliant Blue (w/v), 50% methanol (v/v), destained in 40% methanol (v/v), 10% acetic acid (v/v), and rinsed thoroughly in water. Stained regions of the membrane were excised, kept in 1 M HCl overnight at 22 °C, dried under a flow of argon, and stored at −20 °C.

N-terminal amino acid sequencing was performed on an Applied Biosystems model 470A Sequenator equipped with an on-line model 120A phenylthiohydantoin amino acid analyzer.

Isolation of the NADPH-P450 Oxidoreductase—The NADPH-P450 oxidoreductase remained bound to the DEAE-Sepharose FF/S-100-Sepharose column during the elution with buffer B and was subsequently eluted by augmenting buffer B with 0.5 mM KCl. The reductase was isolated and purified to homogeneity (Fig. 2) on a column of 2.5× ADP-Sepharose 4B (Pharmacia) as previously reported (33) and concentrated to a final activity of 15 units/ml (34).

Reconstitution of Obtusifoliol 14α-Demethylase—The reconstitution mixtures contained 100–150 μl of isolated and dialyzed obtusifoliol 14α-demethylase (50–75 pmol), 50–100 μl of NADPH-P450 oxidoreductase (0.75–1.5 units), 30 μl of DLPC (10 mg/ml sonicated as described in Ref. 33), and 30 μl of obtusifoliol (500 μM) mixed in a glass vial in the given order by repeated resuspension. The reaction was initiated by the addition of 15 μl of NADPH (25 mg/ml), and the volume was adjusted to 300 μl with 50 mM Tricine, pH 7.9. The sealed samples were incubated under constant gentle agitation (30 °C, 4 h), after which the reaction was stopped with 400 μl of 10% KOH (w/v) in MeOH. The substrate and product were extracted into hexane (2 × 1 ml), and the combined hexane phases were evaporated to dryness before the sample was silylated by heating with N,N-bis-trimethylsilyl-trifluoroacetamide for 30 min at 100 °C and analyzed by GC-MS. The GC-MS system consisted of an HP5890 Series II gas chromatograph equipped with an HP-1 column (25 m × 0.2 mm; 0.35 μm film thickness) and directly coupled to a Jeol JMS-AX505W mass spectrometer. The oven temperature was 280 °C for 3 min, 280–290 °C at 1 °C/min, and 290 °C for 30 min. The head pressure was 70 kPa, and the injection temperature was 260 °C. Split injection was applied. The GC-MS interface line was maintained at 250 °C. The spectrometer repetition rate was 1.0 scan/s. The ion source was run in electron impact mode at 260 °C, 70 eV ionization energy.

Computer Analysis—Sequence computer analysis was done using the programs in the GCG Wisconsin Sequence Analysis Package.

RESULTS

Purification of Obtusifoliol 14α-Demethylase—The method used for the isolation of obtusifoliol 14α-demethylase from sorghum microsomes relies on an initial ion exchange step to provide a P450 eluate devoid of yellow microsomal pigments, NADPH-P450 oxidoreductase, and cytochrome b₅. The individual P450s present in the eluate were subjected to fractionation by dye column chromatography. The use of a reactive yellow dye column followed by a Cibacron blue dye column permitted the isolation of a single P450 with an apparent molecular mass of 53 kDa as determined by SDS-PAGE (Fig. 2). The isolated enzyme produced an absorption spectrum with maxima at 417, 534, and 563 nm (Fig. 3) characteristic of low spin P450s and a carbon monoxide difference spectrum with a characteristic Soret absorption maximum near 450 nm (Fig. 4), confirming its assignment as a P450. The efficiency of the individual steps in the isolation procedure is apparent from the Coomassie Blue-stained SDS-PAGE gel and the corresponding immunoblot shown in Fig. 2.

The presence of detergents in all buffers hampered accurate determination of protein in the fractions. Accordingly the increase in specific activity during the course of the isolation (Table I) was calculated based on the total content of P450 as...
determined from the carbon monoxide difference spectra and the content of the isolated P450 determined semiquantitatively by scanning the immunoblot (Fig. 2). This method allows a rough estimation of the content of the isolated P450 in the different fractions and shows that the isolated P450 constitutes between 15 and 25% of the total P450 content of sorghum microsomes. The total content of P450 in the microsomal membranes is 0.2 nmol/mg of protein (32), and the isolated P450 thus constitutes about 0.2% of the total microsomal protein. The microsomal fraction was obtained by differential centrifugation (31). Immunoblot analysis of the chloroplast and mitochondrial pellets and of the postmicrosomal supernatant revealed that the isolated P450 is confined to the microsomal membranes (data not shown).

For amino acid sequencing, the isolated P450 was subjected to preparative SDS-PAGE. A portion of the electroeluted protein was digested with endoproteinase Lys-C or endoproteinase Glu-C. The generated fragments were separated by SDS-PAGE, transferred to ProBlott membranes, and subjected to amino acid sequencing together with the uncleaved protein (Table II). The databases were searched for sequences showing similarity to the partial amino acid sequences obtained. In the BLAST search (35) with the AAGAFSYISFGGGRH sequence (Table II, fragment 1), the P450s that showed the highest sequence similarity were all 14α-demethylases from mammals and yeasts, which indicates that the isolated P450 is a sterol 14α-demethylase. The cDNAs encoding the lanosterol 14α-demethylase from rat (9, 10) and several yeast species (11–13) have been cloned. All the sequences have been assigned to the CYP51 gene family (36).

A unique feature for the CYP51 family is a substitution in the heme binding domain of a highly conserved arginine with a histidine residue (9). Fragment 1 aligns with this domain, and the last amino acid of the fragment is the unique histidine, which supports the hypothesis that the isolated P450 is the plant sterol 14α-demethylase. The substrate for the plant 14α-demethylase is obtusifoliol (22–24). We subsequently investigated the ability of the isolated enzyme to bind sterols and to metabolize obtusifoliol.

Substrate Binding—The isolated P450 (122 pmol in 270 μl) was used for substrate binding experiments testing putative substrates at a final concentration of 50 μM. Obtusifoliol produced a Type I substrate binding spectrum (29) with a maximum at 390 nm and a minimum at 418 nm (Fig. 5a). Neither lanosterol (Fig. 5d), the natural substrate for the 14α-demethylase in yeast and mammals (37), nor the three common plant sterols campesterol (Fig. 5b), sitosterol (Fig. 5c), and stigmasterol (data not shown) produced substrate binding spectra.

The Type I binding spectrum obtained with obtusifoliol represents a shift of the P450 from a low spin to a high spin state. In a complete transition from a low spin to a high spin state the theoretical absorption coefficient $\varepsilon_{390-420}$ is 138 M$^{-1}$ cm$^{-1}$ (29). From the obtusifoliol binding spectrum an absorption coefficient $\varepsilon_{390-420}$ of 8 M$^{-1}$ cm$^{-1}$ is calculated. Thus the magnitude of the obtusifoliol binding spectrum obtained is only 6% of that expected from a full transition, which indicates that the isolated P450 is present in an apparent spin state equilibrium. A similar low conversion from low to high spin state was obtained by Yoshida and Aoyama (2) upon the addition of saturating amounts of lanosterol to isolated yeast 14α-demethylase, which could imply that the low spin state conversion is a general feature of 14α-demethylases or that isolation procedures involving detergent solubilization results in the
isolation of partially inactivated 14α-demethylases.

**Reconstitution**—For a plant P450 to exert its catalytic activity as a monooxygenase it needs to interact with NADPH-P450 oxidoreductase. The two membrane proteins form a short electron transport chain in which reducing equivalents from NADPH are transferred via the NADPH-P450 oxidoreductase to the P450 which binds the substrate and activates molecular oxygen. Accordingly, reconstitution of a P450 monooxygenase requires its insertion into a lipid micelle together with the NADPH-P450 oxidoreductase. The micelles in the present study were prepared from DLPC and were incubated with NADPH and obtusifoliol. At the end of the incubation period (4 h) the sterols present were extracted into hexane, silylated, separated by gas chromatography, and their structures were determined by mass spectroscopy (Fig. 6).

In control experiments carried out in the absence of NADPH-P450 oxidoreductase, obtusifoliol was not metabolized. The TMS derivative of obtusifoliol has a retention time of 24.5 min (Fig. 6a). In the presence of NADPH-P450 oxidoreductase a product with a retention time of 25.0 min clearly separated from obtusifoliol was formed (Fig. 6b). Product formation was also dependent on the presence of NADPH (data not shown).

**TABLE II**

| Fragment | Sequence |
|----------|----------|
| 1        | AAGAFSYISFGGRH |
| 2        | GRPTTE      |
| 3        | KFGPEGK     |
| 4        | KPLAXIR     |
| 5        | YRFNVPFTFGPUGVFVH/XY |
| 6        | MDLADIPQ/KQQRIMGAXALVV |

**Fig. 5.** Substrate binding spectra of obtusifoliol 14α-demethylase. Each cuvette contained 270 μl of P450 (122 pmol). Sterols (30 μl) as indicated below were prepared to 500 μM in 1% Tween 80 and added to the measuring cuvette. 1% Tween 80 in water was added to the reference cuvette. a, obtusifoliol; b, campesterol; c, sitosterol; d, lanosterol. The obtusifoliol binding spectrum was recorded at a scan speed of 1 nm/s, and the remaining spectra were recorded at 2 nm/s. All spectra were corrected for the absorbance contribution of the standard.

**Fig. 6.** The catalytic properties of reconstituted obtusifoliol 14α-demethylase as analyzed by GC-MS using obtusifoliol as a substrate. Isolated obtusifoliol 14α-demethylase was reconstituted with NADPH-P450 oxidoreductase in DLPC micelles. Sterols were extracted into hexane, silylated, and subjected to GC-MS analysis. a, GC profile of control experiment in which the reconstitution was carried out in the absence of NADPH-P450 oxidoreductase. Obtusifoliol has a retention time of 24.5 min. The inset shows the complete elution profile to document the purity of the obtusifoliol preparation obtained from Dr. Akihisa. b, GC profile of a reconstitution experiment demonstrating the metabolism of obtusifoliol. The product has a retention time of 25.0 min. The inset shows that no other product was formed. c, mass spectrum of silylated obtusifoliol showing the molecular ion of m/z 498. d, mass spectrum of the silylated product formed from obtusifoliol having a molecular ion of m/z 482. The spectrum identifies the product as 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol.
The mass spectrum of the TMS derivative of obtusifoliol (Fig. 6c) has a molecular ion of $m/z$ 498. The TMS derivative of the product has a molecular ion of $m/z$ 482 (Fig. 6d). This molecular ion is 16 mass units smaller than that of obtusifoliol and would suggest the loss of a methyl group and the introduction of a double bond. In the mass spectrum of TMS-obtusifoliol, the intensity of $M^-\text{CH}_3$ and $M^-\text{CH}_2$-TMSOH is larger than that of the molecular ion. In contrast, the molecular ion has the highest intensity in the mass spectrum of the silylated product. This feature is indicative of the removal of a methyl group at either the C-10 or C-14 position (5, 37). The gas chromatography retention time of the demethylated metabolite is larger than that of obtusifoliol (Fig. 6b), which demonstrates that the methyl group has been removed from the C-14 position (5, 38). The same characteristic mass spectral fragmentation pattern and intensity ratios for substrate and metabolite have been obtained with microsomes from Saccharomyces cerevisiae (5) and maize (22) and have been ascribed to the activity of lanosterol 14α-demethylase and obtusifoliol 14α-demethylase, respectively. We thus conclude that the metabolite formed is 4α-methyl-5α-ergosta-8,14,24(28)-tri-en-3β-ol (Fig. 1, 2), the 14α-demethylated obtusifoliol with a double bond introduced at Δ14.

Based on the substrate binding properties of the isolated P450 and the activity of the reconstituted monooxygenase, we conclude that the isolated P450 is obtusifoliol 14α-demethylase.

**DISCUSSION**

In the present study we report the isolation and reconstitution of the P450 responsible for plant sterol 14α-demethylase. The single product obtained with the reconstituted enzyme is 4α-methyl-5α-ergosta-8,14,24(28)-tri-en-3β-ol, which is the 14α-demethylated product of obtusifoliol with a double bond introduced at the Δ14 position. The obtusifoliol 14α-demethylase thus catalyzes a reaction analogous to the sterol 14α-demethylases of nonphotosynthetic phyla, although it uses a different substrate and has higher substrate specificity (23, 37). We show that the isolated obtusifoliol 14α-demethylase is a low spin P450, which produces a Type I binding spectrum with obtusifoliol and not with any of the other sterols tested. The high substrate specificity of the obtusifoliol 14α-demethylase observed in the substrate binding experiments corresponds well with the results obtained with microbial membranes from maize, which were found to catalyze the demethylation of obtusifoliol but not to metabolize lanosterol, dihydrolanosterol, or eburicol (22, 23). The microsomal membranes from maize catalyze the additional formation of 24-methylene lophenol, which most likely reflects the presence of a Δ14-sterol reductase in the microsomal preparation (21).

cDNA clones encoding several mammalian and fungal 14α-demethylases have been isolated. Sequence and function similarities have placed them in the CYP51 family, which is unique in being the only P450 family to occur in different phyla (36). One of the partial amino acid sequences obtained for obtusifoliol 14α-demethylase in the present study indicates that this P450 should also be placed in the CYP51 family. This sequence is AAGAFSYISFGGGRH and partly covers the conserved heme binding region, being only two amino acid residues short of the cysteine residue, which serves as the fifth ligand to the heme group. Within the CYP51 family, the heme binding region is highly conserved with the consensus sequence FGXXGRHRKCGEXFPAY, where X refers to any amino acid (9–13). This sequence diverges from the universal consensus sequence in the P450 supergene family by a histidine substitution of the highly conserved arginine residue present in almost all other eukaryotic P450s. The same unique substitution is observed in the sequence from the sorghum enzyme.

Plant P450s are difficult to isolate mainly due to their temporal expression and their presence in low amounts (39, 40). Plant sterols are produced over long developmental periods by a biosynthetic machinery which is nonabundant (41). This is reflected in the present study where the obtusifoliol 14α-demethylase was found to constitute approximately 0.2% of the microsomal protein in the sorghum seedlings. In yeast, rats, and humans the level is 10–30 times higher (2, 3, 8). In this study we have taken advantage of the ability of dye affinity columns to separate individual P450s present in sorghum microsomes, an approach previously applied for the isolation of the multifunctional tyrosine N-hydroxylase (CYP79) involved in the biosynthesis of the cyanogenic glucoside dhurrin in sorghum (32). Like the obtusifoliol 14α-demethylase, the tyrosine N-hydroxylase constitutes about 20% of the total P450 present in the membranes.

CYP51 from humans was isolated by immunoaffinity chromatography (8) using an antibody toward CYP51 isolated from pig (7). The polyclonal antibody raised against obtusifoliol 14α-demethylase in the present study showed only weak cross-reactivity toward microsomal preparations from other plants (data not shown) and would thus not be expected to cross-react with 14α-demethylases from other phyla. Similarly, antibodies against CYP51 isolated from yeast (5, 26) did not cross-react with Nicotiana tabacum proteins. This suggests that isolation of plant CYP51 using immunoaffinity chromatography would not be generally feasible.

The reconstituted obtusifoliol 14α-demethylase exhibits low catalytic activity. The reconstitution process involves the combined insertion of isolated obtusifoliol 14α-demethylase and isolated NADPH-P450 oxidoreductase into artificial membranes. In the present study, the artificial membranes were prepared using DLPC, since among a range of different lipids and lipid mixtures tested, DLPC was found to give the highest reconstitution rate with CYP79, a P450 enzyme likewise isolated from sorghum microsomes (33). The inclusion of cytochrome b5 and NADH-cytochrome-b5 oxidoreductase into the artificial membranes may also be essential for increased catalytic activity. However, Trzaskos et al. (3) found no significant effect of cytochrome b5 and NADH-cytochrome-b5 oxidoreductase on the reconstitution of CYP51 isolated from rat liver microsomes. The most likely explanation for the low catalytic activity of the reconstituted obtusifoliol 14α-demethylase is the residual and variable amounts of detergents which are introduced in the reconstitution assays upon addition of the detergent solubilized 14α-demethylase and oxidoreductase. An inhibitory effect of residual amounts of detergents has been reported in the reconstitution of the 14α-demethylase isolated from Candida albicans (6). Finally, for unknown reasons, some P450s like those belonging to the P450 3A subfamily are recalcitrant to reconstitution (42). Using recombinant P450 3A4 produced in Escherichia coli cells, Yamazaki et al. (43) identified a number of additional parameters (buffers, divalent cations, glutathione) which may be of importance in the reconstitution process. Due to the minute amounts of obtusifoliol 14α-demethylase which can be isolated from sorghum seedlings, we have not been able to carry out a detailed study to identify the factors necessary to achieve optimal reconstitution of obtusifoliol 14α-demethylase.

Based on the antibody and the amino acid sequence data obtained, studies are in progress to clone the gene encoding obtusifoliol 14α-demethylase from sorghum. This will serve to clarify whether the obtusifoliol 14α-demethylase indeed belongs to the CYP51 family and thus is the first plant P450 to belong in a nonplant P450 family. Expression of the cDNA clone in E. coli as already achieved for CYP79 (44, 45) will
permit the generation of large amounts of enzyme and thus a
detailed study of its kinetic parameters.

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