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Interactions of Avocado (*Persea americana*) Cytochrome P-450 with Monoterpenoids

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ABSTRACT

The microsomal fraction of avocado (*Persea americana*) mesocarp is a rich source of cytochrome P-450 active in the demethylation of xenobiotics. Cytochrome P-450 from this tissue has been purified and well characterized at the molecular level (DP O'Keefe, KJ Leto [1989] Plant Physiol 89: 1141–1149; KR Bozak, H Yu, R Sirevag, RE Christoffersen [1990] Proc Natl Acad Sci USA 87: 3904–3908). Despite this extensive characterization, the role of the enzyme *in vivo* was not established. Optical and electron paramagnetic resonance binding studies described here suggest that the monoterpenoids, nerol and geraniol, are substrates of avocado cytochrome P-450 (spectral dissociation constant of 7.2 and 35 micromolar, respectively). Avocado microsomes have been shown to catalyze the hydroxylation of these monoterpenoids, and both nerol and geraniol have been shown to inhibit the activity of avocado cytochrome P-450 toward the artificial substrate 7-ethoxycoumarin, with nerol a competitive inhibitor of this activity.

Cyt P-450 enzymes are membrane-associated hemoprotein monooxygenases that are involved in a number of biosynthetic (29) and detoxification (3, 15) pathways in plants. Because of their importance in xenobiotic and drug metabolism, these enzymes have been thoroughly studied in mammalian liver (4), with mechanistic and structural details based to a large extent on studies of the bacterial (*Pseudomonas putida*) camphor hydroxylase system (23). Although considerable progress has been made in characterizing mammalian and bacterial Cyt P-450 enzymes, much less is known about the plant enzymes. This is due principally to the low amounts of Cyt P-450 present in plant tissues, and the apparent lability of the enzyme following detergent solubilization (7, 12, 15, 25, 29).

Recently, however, Cyt P-450 active in the demethylation of the artificial substrate (pCMA[1]) was purified from avocado mesocarp tissue (16). Two immunologically similar polypeptides, of molecular mass 47 and 48 kD, were isolated. Avocado mesocarp has long been known to be a rich source of Cyt P-450 active in the demethylation of pCMA and other xenobiotics (6, 16, 28). However, the natural substrate of the avocado Cyt P-450 was not identified. A ripening-related cDNA from avocado mesocarp has been sequenced that bears significant homology to mammalian Cyt P-450 sequences and codes for an N-terminal amino acid sequence identical to that obtained with purified avocado Cyt P-450 (1).

Thus, avocado Cyt P-450 is one of the best characterized enzymes of its class in plants. Despite this extensive characterization, the role of the enzyme *in vivo* remains unclear. In this communication, we present evidence that avocado mesocarp contains Cyt P-450 active in the hydroxylation of the monoterpenoids nerol and geraniol. This activity is well characterized as a Cyt P-450-dependent reaction in the plant *Catharanthus roseus* (12, 14), where the hydroxylation of geraniol initiates the biosynthesis of iridoid monoterpenes and the indole alkaloids.

MATERIALS AND METHODS

Avocados (*Persea americana* cv Hass) were purchased locally and ripened at room temperature. Clotrimazole, lauric acid, umbelliferone, 7-ethoxycoumarin, NADPH, and glucose 6-phosphate dehydrogenase were from Sigma. DMSO, pCMA, *t*-cinnamic acid, nerol (99%), and geraniol (99%) were from Aldrich. pCMA was purified by recrystallization as the HCl salt. Kaurene, purified from foliage of *Cryptomeria japonica,* was a gift of Professor J.R. Bowyer, Royal Holloway & Bedford New College, University of London, UK.

Microsomes were routinely prepared from 500-g batches of mesocarp tissue as previously described (16) and resuspended in 0.1 M Mops-NaOH, pH 7.0, 20% (v/v) glycerol to 25 mL (6.0 mg protein/mL). Partial purification of Cyt P-450 was achieved as follows. Microsomes were concentrated by centrifugation at 100,000*g* and resuspension to 4 mL in 0.1 M Mops-NaOH, pH 7.0, 20% (v/v) glycerol. An equal volume of 4% (v/v) Triton X-100 in the same buffer was added slowly, with stirring. After being stirred for 45 min at 4°C, the solution was centrifuged at 100,000*g* to remove unsolubilized material. The supernatant was applied to a 2 × 10 cm column of DEAE-Sephacel equilibrated with 0.2% (v/v) Triton X-

[1] Abbreviations: pCMA, 4-chloro-*N*-methylaniline; ECOD, 7-ethoxycoumarin deethylase; 7-EC, 7-ethoxycoumarin; EPR, electron paramagnetic resonance; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; K_s, spectral dissociation constant.

100, 20% (v/v) glycerol, 10 mM potassium phosphate, pH 7.0. After washing the column with 30 mL of the equilibration buffer, Cyt P-450 was eluted by application of a 50 to 500 mM linear KCl gradient (100 mL). Fractions of 3 mL were collected and absorbance at 405 nm monitored. The Cyt-containing fractions were concentrated by precipitation with 80% saturated ammonium sulfate, redissolved in 0.1% (w/v) CHAPS, 10% (v/v) glycerol, 0.1 M potassium phosphate, pH 7.5, and desalted by passage through a Sephadex G-25 column. Solubilized Cyt P-450 was concentrated for EPR spectrometry using Centricon 10 ultrafilters (Amicon).

[^{3}H]Geraniol (0.04 mCi/μmol) and [^{3}H]nerol (0.05 mCi/μmol) were prepared by sodium boro[^{3}H]hydride (Amersham) reduction of the corresponding aldehydes, prepared from the alcohols by oxidation with active MnO_2. Monoterpenoids were purified by TLC on silica gel plates impregnated with 5% $AgNO_3$. 10-Hydroxygeraniol and 10-hydroxynerol were synthesized from geranyl or neryl acetate by selenium dioxide and *t*-butylhydroperoxide oxidation (22) to provide a mixture of 10-hydroxy- and 10-oxo-geranyl and neryl acetates. The mixtures were converted to the diols by reduction with lithium aluminium hydride in diethyl ether. Diols were purified by column chromatography on silica gel, with increasing concentration of diethyl ether in hexane as the eluting solvent. Structures were confirmed by NMR spectrometry.

For assay of monoterpenoid hydroxylase activity, microsomes (0.5–1.0 mg protein) were incubated in the presence of 0.15 unit glucose 6-phosphate dehydrogenase, 100 mM glucose 6-phosphate, 0.5 mM NADPH, 1 mM DTT, 0.1 M potassium phosphate, pH 7.5, in a volume of 1 mL. After equilibration at 30°C, the reaction was initiated by addition of 0.5 μCi [^{3}H] geraniol or [^{3}H]nerol in acetone. The reaction was stopped by addition of 0.5 mL methanol, and after addition of the appropriate carrier diol (10 μg), the mixtures were extracted with dichloromethane. Products were separated by TLC on Whatman LK6DF silica gel plates in dichloromethane-methanol (92:8, v/v). Marker diol was visualized with iodine vapor, the radioactive diol bands scraped off, and counted by liquid scintillation spectrometry. Addition of carbon monoxide, where required, was by gastight syringe into assay tubes sealed with serum stoppers.

ECOD activity was routinely assayed in the presence of 250 μM 7-EC as described by Werck-Reichhart *et al.* (28) using a Perkin-Elmer 3000 fluorimeter to record fluorescence change at 460 nm as a function of time, with an excitation wavelength of 380 nm. Difference spectroscopy (9) was performed using a Cary 210 spectrophotometer. EPR spectrometry was performed at cryogenic temperatures using a JEOL RE1X spectrometer fitted with an Oxford Instruments liquid helium cryostat. Spectra were recorded and manipulated using a Dell microcomputer running ASYST software. Characteristic lineshapes were established using several sets of samples, consisting of 3 to 6 nmol Cyt P-450. The conditions used for EPR were microwave power 1 mW, temperature 14 K, modulation width 1.25 mT (low spin heme); or microwave power 10 mW, temperature 5 K, modulation width 1.25 mT (high spin heme). Protein was estimated using a modified Lowry procedure (13).

Denaturing PAGE was performed using 7.5 to 15% (w/v) acrylamide gradient gels (15 cm in length) in the presence of SDS as described by Laemmli (11). Protein mol wt markers were obtained from Sigma (MW-SDS-70L kit). Western blotting was performed as described by Towbin *et al.* (26). Anti-ARP 1 serum was a kind gift of Dr. D.P. O'Keefe, E.I. DuPont de Nemours and Company, (Wilmington, DE). Visualization of antigen on western blots was achieved using an ExtrAvidin kit (Sigma).

RESULTS AND DISCUSSION

Difference Spectroscopy of Avocado Microsomes

The binding of compounds to Cyt P-450 may be monitored using difference spectroscopy (9) because substrate binding results in a shift in the Soret maximum of the Cyt. On binding of a substrate, a type I difference spectrum is typically obtained, with a peak at 390 nm and trough at 420 nm (9). A type I binding spectrum was obtained with avocado microsomes after addition of pCMA, as previously described (5, 16), but could not be obtained with 7-EC. These compounds serve as model substrates, undergoing demethylation and deethylation, respectively, in the presence of catalytically competent Cyt P-450 (6, 16, 28). Of possible natural substrates of plant Cyt P-450, we examined the compounds kaurene, lauric acid, oleic acid, ferulic acid, *t*-cinnamic acid, nerol, and geraniol for their ability to induce such spectra with avocado microsomes. Of the compounds tested, only nerol, its isomer geraniol, and lauric acid were found to induce formation of a type I difference spectrum (Fig. 1), with an absorbance peak centered at 390 nm and a trough at 425 nm. This effect was not seen with *t*-cinnamic acid, kaurene, oleic acid, or ferulic acid at similar concentrations. Titration of microsomes with nerol (Fig. 2A) or geraniol (not shown) yielded data that could be fitted to linear double reciprocal plots. Figure 2B shows that the apparent K_s for nerol binding to the membrane-bound Cyt P-450 was 7.2 μM. The K_s for geraniol was similarly determined to be 35 μM. These values are considerably lower than those previously found for pCMA (180–378 μM) (5, 16).

Titration with lauric acid yielded a K_s of 350 μM, as was previously obtained (5), which was considerably higher than the values determined for nerol and geraniol. To determine whether binding of monoterpenoids and lauric acid could be due to multiple Cyt P-450 isoforms, the spectral response of microsomes saturated with nerol to the subsequent addition of lauric acid was examined (9). It was found that addition of lauric acid (up to 1 mM) to microsomes pretreated with nerol (1 mM) did not induce a spectral response. Thus, lauric acid may bind to the same Cyt P-450 isoform involved in the interaction with nerol, but with considerably lower affinity. However, the possibility that a distinct lauric acid hydroxylase (20, 21) may bind nerol at the high concentration employed cannot be discounted.

If an extinction coefficient of 126 $mM^{-1}cm^{-1}$ was assumed for the difference in absorbance between 390 and 425 nm at saturating levels of monoterpenoid (2), the monoterpenoid-binding Cyt accounted for between 55 and 78% of Cyt P-450 detectable as the ferrous-CO complex in avocado microsomes.

The hydroxylation of geraniol and nerol is well characterized as a Cyt P-450-catalyzed activity in *Catharanthus roseus* microsomes (12, 14); thus, the spectral data indicated that a

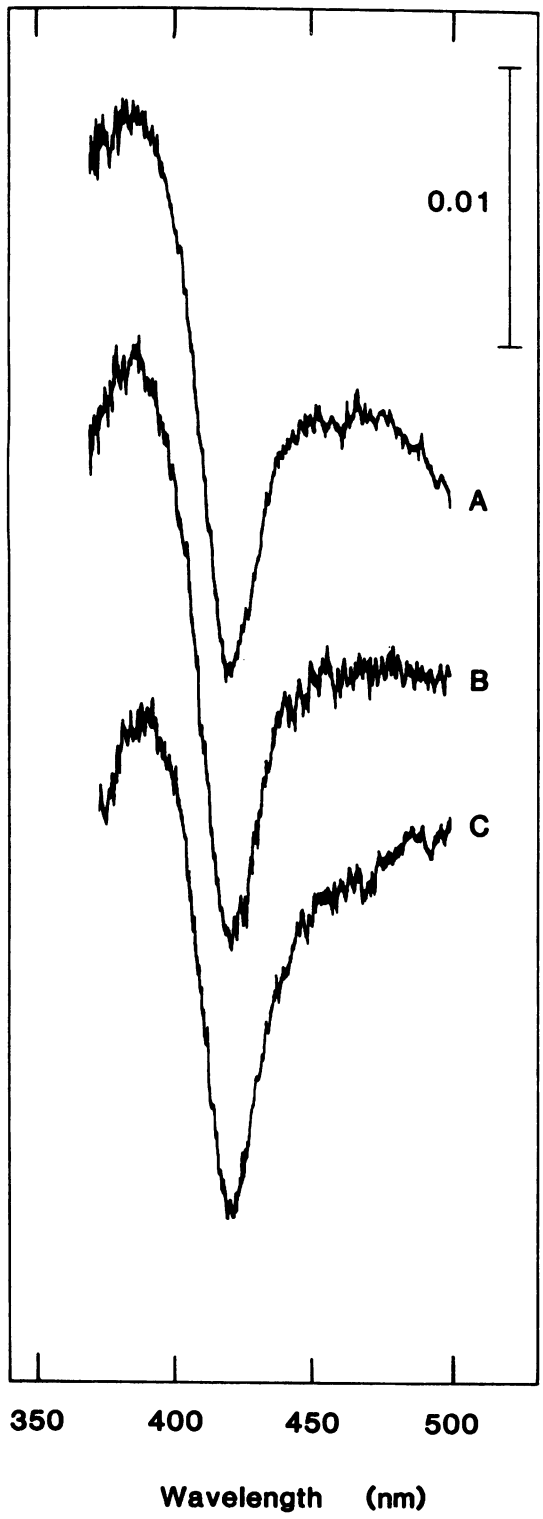

Figure 1. Interaction of nerol, geraniol, and lauric acid with Cyt P-450 in microsomes of *P. americana*. Avocado microsomes were suspended to a protein concentration of 1 mg/mL, and a baseline spectrum recorded between 500 and 370 nm. The difference spectra resulting from addition of 0.4 mM nerol (A), 0.4 mM geraniol (B), and 0.5 mM laurate (C) are shown. All additions were made in DMSO.

similar activity might be catalyzed by Cyt P-450 from avocado mesocarp.

EPR Studies

Changes in the visible absorption spectrum of Cyt P-450 on interaction with substrates or inhibitors has been shown to correlate with changes in the spin state of the heme iron detected using EPR (9, 17, 24).

Microsomes at 14 K gave EPR signals characteristic of oxidized low-spin Cyt P-450 at g = 2.42, g = 2.25, and g = 1.915 (Fig. 3A). At 5 K, signals from high-spin heme at g = 7.6 and g = 4.13 were observed (Fig. 4), showing that both spin state forms occur in membrane-bound avocado Cyt P-450. Both low- and high-spin signals were removed on reduction of the microsomes with dithionite. The mixed spin state may represent the contributions of different Cyt P-450 isoforms or the presence of a single species in which the low- and high-spin states are sufficiently close in energy for an equilibrium to occur (9).

Addition of the type I ligands, nerol and geraniol, produced g-value shifts and lineshape changes in both low- and high-spin signals, giving a major shift to the low-spin spectrum (Figs. 3, C and D, and 5, C and D). The addition of nerol or geraniol induced a shift of the remaining high-spin peak to g = 7.7, and additional resonances were observed in the low-spin spectra near g = 1.9 and between g = 2.4 and g = 2.5. Similar but less marked effects were observed after the addition of pCMA (not shown), but the addition of lauric acid (Figs. 3B and 5B) or *t*-cinnamic acid (not shown) gave only a slight increase in the high-spin form of the Cyt. The addition of clotrimazole, which is a type II ligand of avocado Cyt P-450 (not shown), caused the conversion of Cyt P-450 to a low-spin form with distinct g values (g = 2.5, g = 2.27, and g = 1.915) and lineshape (not shown).

These results clearly demonstrate an interaction between Cyt P-450 and the ligands nerol and geraniol, but the spin state changes observed with these monoterpenoids are at first sight difficult to reconcile with the optical absorption measurements detailed above. EPR studies on potato microsomes (19) showed that addition of 0.1 mM type I ligand *t*-cinnamate resulted in a slight increase in the high-spin form of Cyt P-450, with a corresponding decrease in the low-spin form. Addition of 10 mM type II ligand, aniline, produced the opposite effect by decreasing the high-spin and increasing the low-spin signals. This latter effect was clearly seen only in tulip bulb microsomes. These results confirmed the evidence of Rich *et al.*'s (19) optical binding studies that ligands alter the spin states of plant Cyt P-450 heme, and confirmed the optical assignment of spin states.

The effects of *t*-cinnamate binding in our study and another (19) are similar to those of lauric acid in this study, and can be ascribed to type I ligands of low affinity. The high-affinity ligands, nerol and geraniol, produce much more dramatic spectral changes, although the induced spin state changes are in apparent contradiction to those indicated by absorption measurements and previous work (19). However, Peisach *et al.* (18), in a study of Cyt P-450 from *Rhizobium japonicum*, revealed that in samples treated with the type I ligand, phenobarbital, the spin state of the heme iron was temperature

dependent, shifting the optical spectrum from high spin to low spin at cryogenic temperatures. This was confirmed by EPR measurements. Phenobarbital binding to *Rhizobium* Cyt P-450 produced similar spin-state changes, as detected by EPR, as were observed in this study with nerol and geraniol, and also gave rise to new low-spin resonances. Therefore, we conclude that the EPR study at cryogenic temperatures and the optical experiments at physiological temperatures cannot be compared due to these temperature-dependent spin-state changes of the avocado Cyt P-450.

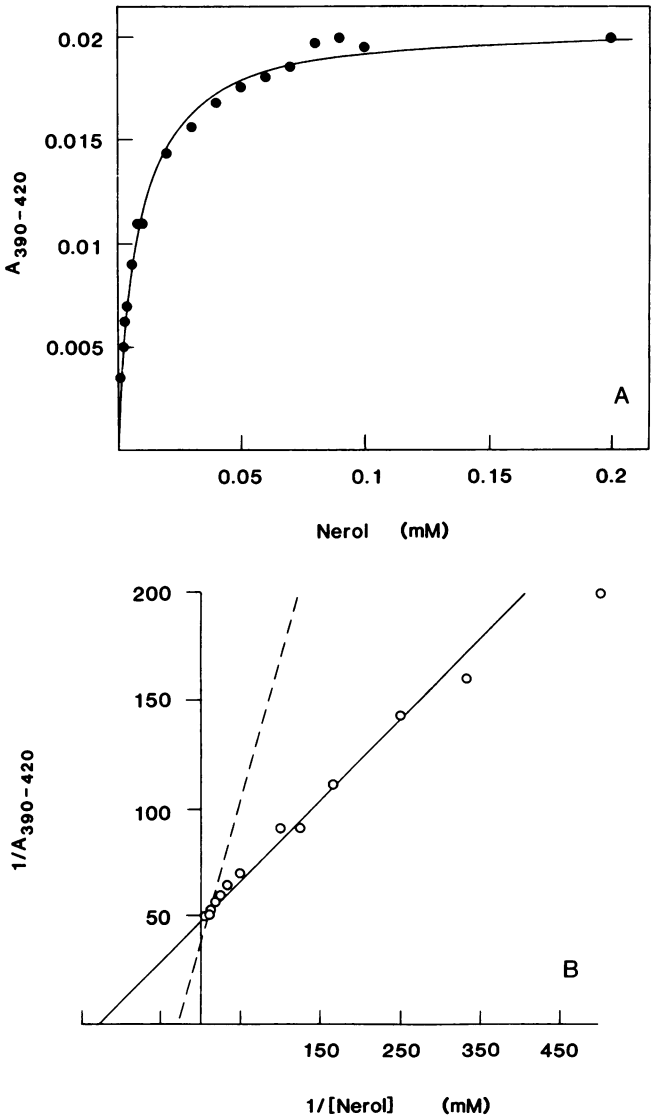

Figure 2. Titration of the spectral response of *P. americana* Cyt P-450 to monoterpenoids. A, Difference spectra of avocado microsomes (1 mg/mL protein) were recorded following addition of increasing amounts of nerol, and absorbance changes plotted as a function of nerol concentration. B, Double-reciprocal plot of the data shown in A. The dashed line represents the line of best fit to similar data derived from titration with geraniol.

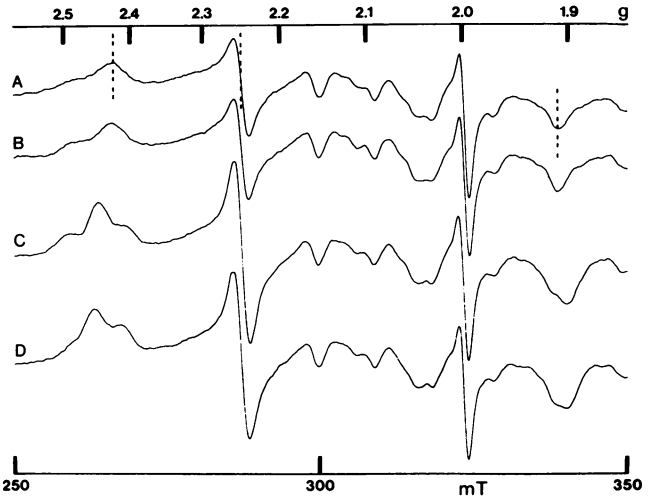

Figure 3. EPR spectra of oxidized *P. americana* microsomes; effect of ligands on low-spin Cyt P-450 signals. Avocado microsomes were suspended in 0.1 M potassium phosphate, pH 7.0, 20% (v/v) glycerol, and 1 mM EDTA to a concentration of 20 nmol Cyt P-450/mL. Spectra were recorded on 0.3 mL samples. Additions were made in 3 μL DMSO: A, DMSO alone; B, 1.0 mM laurate; C, 1.0 mM nerol; D, 1.0 mM geraniol. Conditions of measurement are described in "Materials and Methods." Dashed lines indicate g = 2.42, g = 2.25, and g = 1.915.

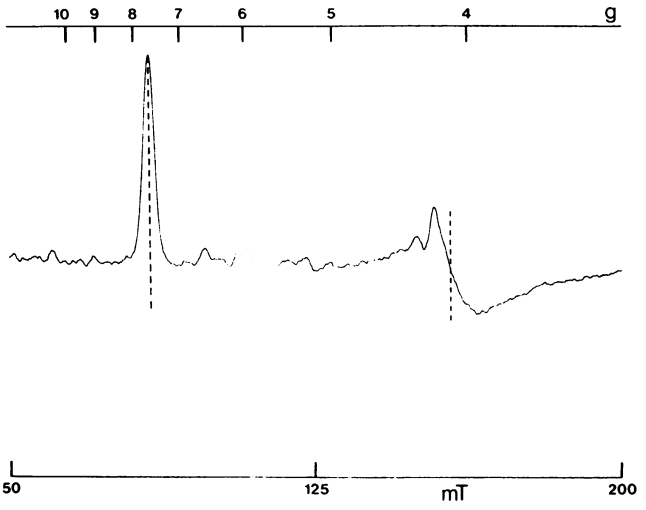

Figure 4. EPR spectrum of *P. americana* microsomal Cyt P-450 (high spin). Avocado microsome samples were prepared for EPR as described in the legend to Figure 3. The spectrum shown is the result of subtracting the spectrum of dithionite-reduced microsomes from that of oxidized microsomes. Conditions of measurement are described in "Materials and Methods." Dashed lines indicate g = 7.6 and g = 4.13.

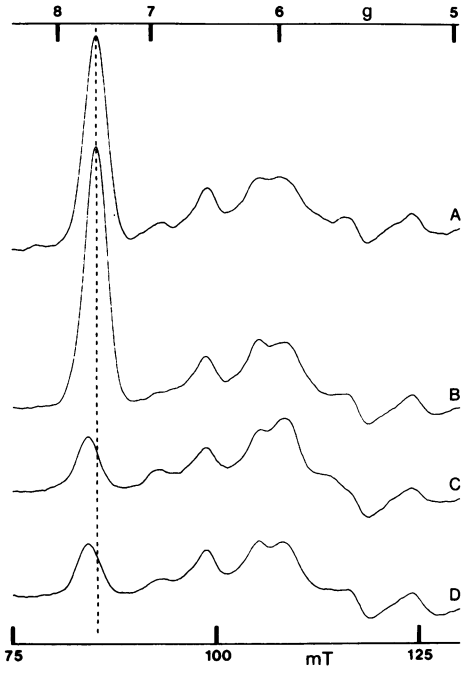

Figure 5. Effect of ligands on the g = 7.6 EPR signal of *P. americana* microsomes. Samples of avocado microsomes were prepared for EPR as described in the legend to Figure 3. Additions were: A, DMSO; B, 1.0 mM laurate; C, 1.0 mM nerol; D, 1.0 mM geraniol. Conditions of measurement are described in "Materials and Methods." The dashed line indicates g = 7.6.

Solubilized Cyt P-450

As previously reported (16), avocado Cyt P-450 is effectively solubilized by Triton X-100 treatment of microsomes. Solubilized Cyt P-450 was partially purified to a specific activity of 2.1 nmol/mg protein. This preparation could be exchanged into the zwitterionic detergent CHAPS by precipitation with ammonium sulfate and gel filtration with minimal loss of Cyt. In the absolute absorption spectrum of partially purified Cyt in Triton X-100, the Soret (gamma) band was found to be at approximately 410 nm, typical of a low-spin ferricytochrome P-450. After replacement of Triton X-100 with CHAPS, the Soret maximum appeared at approximately 420 nm. These results contrast with those obtained by O'Keefe and Leto (16), and might be explained by a particular effect of reduced Triton X-100 on the spin state of the heme. Addition of nerol or geraniol to the enzyme, in Triton X-100, did not result in the appearance of a binding spectrum when examined by difference spectroscopy, nor did addition of these compounds affect the absolute spectrum of the protein. In contrast, addition of pCMA to the enzyme resulted in the appearance of a type I binding spectrum, and titration revealed an increase in K_s to 550 μM. EPR spectra of the purified Cyt were similar to those observed with microsomes, with a mixture of low-spin (g = 2.42, g = 2.25, g = 1.91) and high-spin (g = 7.65, g = 4.08) forms being present (Fig. 6). These spectra were also unaffected by the addition of geraniol.

Therefore, it would appear that unless a separate Cyt P-450 isoform is purified by this method, solubilization of the enzyme results in loss of its ability to bind monoterpenoids, but not the model substrate pCMA. However, the fact that the K_s for pCMA binding was increased with the solubilized enzyme indicates that the substrate-binding properties of the enzyme are altered by detergents, as has previously been shown (16).

Enzymatic Activities of Avocado Cyt P-450

When avocado microsomes were assayed for their ability to hydroxylate nerol, significant activity was detected (3.65 nmol diol/mg·h). Activity was also detected with geraniol as substrate, at a similar level (3.45 nmol diol/mg·h). The reactions were linear for up to 30 min under standard assay conditions. Monoterpenoid hydroxylase activity was inhibited in the presence of carbon monoxide, with 70% inhibition of nerol hydroxylation in the presence of an atmosphere containing 50% carbon monoxide.

O-Deethylation of the xenobiotic, 7-EC, by avocado microsomes has been demonstrated (28). To test whether this activity is catalyzed by the same Cyt P-450 isoform involved in monoterpenoid hydroxylation, we examined the ability of geraniol and nerol to inhibit ECOD activity in avocado microsomes. Under standard conditions (28), avocado microsomes catalyzed ECOD activity with a rate of 1.12 nmol/mg·h. The reaction followed Michaelis-Menten kinetics, with an apparent K_m for 7-EC of 36 μM. Table I shows that ECOD activity was significantly inhibited in the presence of the monoterpenoids nerol and geraniol. Activity toward 7-EC was also inhibited in the presence of pCMA, but inhibition by *t*-cinnamic acid was low and only observed at relatively high

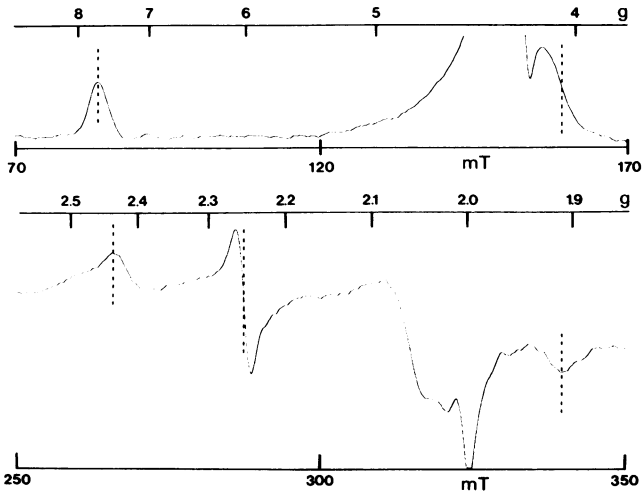

Figure 6. EPR spectra of solubilized *P. americana* Cyt P-450. The EPR spectra of solubilized avocado Cyt P-450 (10 nmol/mL in 0.1 M potassium phosphate, pH 7.5, 0.1% [w/v] CHAPS, 10% [v/v] glycerol, 1.0 mM EDTA) were recorded. Spectra of high-spin (g = 7.65, g = 4.08) Cyt, upper trace, and low-spin (g = 2.42, g = 2.25, g = 1.91) Cyt, lower trace, were obtained as described in "Materials and Methods."

concentrations. Activity was unaffected by the presence of lauric acid.

That the monoterpenoid nerol is a competitive inhibitor of ECOD activity is shown in Figure 7, the Dixon plot yielding a value for K_i of approximately 7.0 μM. This value is very close to that of the apparent K_s derived for the interaction of nerol with the membrane-bound Cyt P-450. The data indicate that monoterpenoid hydroxylation and xenobiotic metabolism in avocado mesocarp microsomes may be catalyzed by the same Cyt P-450 enzyme.

Immunological Studies

Purified avocado Cyt P-450 has been shown to consist of a mixture of two immunologically related polypeptides, ARP-1 and ARP-2, with molecular masses of 47 and 48 kD, respectively, and almost identical N-terminal amino acid sequences (16).

In this study, polyclonal antibodies raised against purified ARP-1 (16) cross-reacted with a single band on western blots of either microsomal proteins or partially purified Cyt P-450 following SDS-PAGE (not shown), with an estimated molecular mass of 53 kD. This result is in close agreement with the estimate of Bozak *et al.* (1) based on the deduced amino acid sequence of the protein, but is in contrast with the observations of O'Keefe and Leto (16), although these authors reported the mass of the native enzyme, as determined by gel permeation HPLC, to be 56 kD. These apparent discrepancies are likely to be due to differences in methodologies employed between different laboratories. This antiserum was not found to inhibit nerol hydroxylation above the level of inhibition found with preimmune serum (data not shown), which might be expected with an antibody raised to denatured protein (16). Western blotting indicates, however, that the avocado Cyt P-450 present in our preparations is likely to be the same species as previously studied in other laboratories, although immunogenic similarity between Cyt P-450 isoforms cannot be discounted (8, 25).

Table I. *Inhibition of ECOD Activity of Avocado Microsomes by Putative Cyt P-450 Substrates*

Activity of avocado microsomes (0.4 mg protein, 0.154 nmol Cyt P-450) was measured as previously described (7) in the presence of the additions shown. One hundred percent activity represents 1.12 nmol umbelliferone formed/mg protein·h. Additions were made in DMSO.

Addition	Concentration	Activity
	mM	
None		100
Nerol	0.2	0.0
	0.1	21.7
	0.001	55.6
Geraniol	0.1	0.0
	0.033	20.0
t-Cinnamate	0.1	73.3
	0.01	100
Lauric acid	0.1	100
pCMA	0.1	34.8

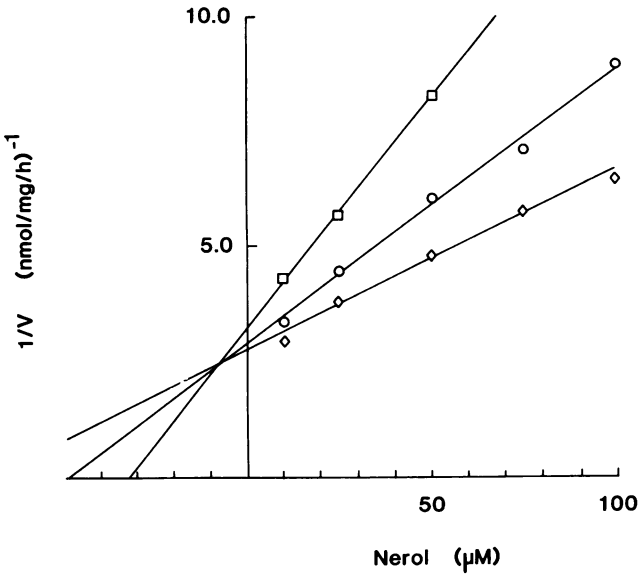

Figure 7. Dixon plot of inhibition of ECOD activity by nerol. Avocado microsomes (0.55 mg protein, 0.23 nmol Cyt P-450) were assayed for ECOD activity in the presence of varying concentrations of nerol with 250 (◇), 187.5 (○), and 125 μM (□) 7-EC.

CONCLUSION

The results described in this paper show that Cyt P-450 in microsomes prepared from ripe avocado mesocarp interacts with the monoterpenoids nerol and geraniol, yielding classic type I substrate-binding optical difference spectra at room temperature. A type I spectral change reflects an increase in high-spin character upon ligand binding (9, 24). The apparent K_s values for this interaction are low, and, especially in the case of nerol, are similar to the K_m values obtained for the hydroxylation of monoterpenoids by Cyt P-450 from other plants (10, 12, 14). In particular, the K_s values for these monoterpenoids were significantly lower than those obtained with pCMA or lauric acid, previously identified as substrates for avocado Cyt P-450.

In high-spin ferric heme proteins, changes to the conformation around the heme can also be detected by EPR at levels far below those required to affect optical spectra. It follows that those ligands that act most strongly on the ferric iron produce the greatest EPR spectral effects. The EPR studies described here showed a strong interaction between the avocado Cyt P-450 heme and the monoterpenoids, nerol and geraniol, although this interaction was lost upon solubilization. As was found with the binding of a type I ligand to *R. japonicum* Cyt P-450 (18), the spin state of the monoterpenoid-liganded heme of avocado Cyt P-450 was temperature dependent. Peisach *et al.* (18) suggested that changes in spin state occurred primarily through effects on the thiolate heme ligand. Perturbation of the sulphur ligand was suggested to occur through the effects of π bonding from the substrate either nearby or through effects on an aromatic amino acid close to the heme. The effect of temperature on spin state may result from structural changes in the protein and/or a

change in the energy equilibrium between spin states on cooling to cryogenic temperatures.

In addition to the spectral data, we have also demonstrated NADPH-dependent monoterpenoid hydroxylation with avocado microsomes, an activity previously attributed to Cyt P-450 in plants (12, 14). The data thus indicate that avocado Cyt P-450 not only interacts spectrally with, but is capable of catalyzing the hydroxylation of, the monoterpenoids, nerol and geraniol. Previous studies have shown that avocado Cyt P-450 is capable of metabolizing a number of xenobiotics. Demethylation of pCMA by both intact microsomes and purified Cyt P-450 of avocado has been demonstrated (6, 16), and microsomes are also active in the deethylation of 7-EC (28) and demethylation of *N,N*-dimethylaniline (8). We have demonstrated the inhibition of ECOD activity by nerol, geraniol, and pCMA, and characterized nerol as a competitive inhibitor of this activity. The data suggest the possibility that all these activities of avocado microsomes might be catalyzed by the same Cyt P-450 species. The catalysis of a number of reactions by a single Cyt P-450 isoform is not unknown, and there is evidence that a single Cyt P-450 isoform may catalyze distinct physiological activities. Recently, for example, both vitamin D 25-hydroxylation and 5β-cholestane-3α,7α,12α-triol 27-hydroxylation were shown unambiguously to be catalyzed by the same Cyt P-450 in liver mitochondria (27). Therefore, it is possible that a single isoform in avocado might be active toward monoterpenoids and the fatty acid, lauric acid, as indicated by the spectral studies described herein. The role of this enzyme in the metabolism of ripening avocado fruit has yet to be identified, and to date there is no evidence available on the presence of monoterpenoids or derived products in avocado fruit. Therefore, we cannot at this stage rule out the possibility that the activity of avocado Cyt P-450 toward monoterpenoids is adventitious. In conclusion, however, the evidence presented suggests that the monoterpenoids, nerol and geraniol, are substrates of avocado Cyt P-450.

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