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# Cytochrome P-450 from the Mesocarp of Avocado (*Persea americana*)

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## ABSTRACT

**The microsomal fraction from the mesocarp of avocado (*Persea americana*) is one of few identified rich sources of plant cytochrome P-450. Cytochrome P-450 from this tissue has been solubilized and purified. Enzymatic assays (*p*-chloro-*N*-methylaniline demethylase) and spectroscopic observations of substrate binding suggest a low spin form of the cytochrome, resembling that in the microsomal membrane, can be recovered. However, this preparation of native protein is a mixture of nearly equal proportions of two cytochrome P-450 polypeptides that have been resolved only under denaturing conditions. Overall similarities between these polypeptides include indistinguishable amino acid compositions, similar trypsin digest patterns, and cross reactivity with the same antibody. The amino terminal sequences of both polypeptides are identical, with the exception that one of them lacks a methionine residue at the amino terminus. This sequence exhibits some similarities with the membrane targeting signal found at the amino terminus of most mammalian cytochromes P-450.**

Cytochrome P-450 dependent monooxygenases are widespread in nature. They are involved in a variety of catabolic and biosynthetic pathways and in the metabolism of drugs and xenobiotics. In plants, Cyt P-450 has been identified, for example, in the *trans*-cinnamic acid and kaurene hydroxylases (10, 11). Others are suspected participants in a number of reactions associated with xenobiotic metabolism (6, 8) (for reviews see Refs. 4 and 20). Within these monooxygenase systems, Cyt P-450 serves as a terminal oxidase, responsible for substrate recognition, binding, and oxygen redox chemistry (5, 29).

The mechanistic and structural details of Cyt P-450 dependent monooxygenases have been developed largely from studies of the bacterial (*Pseudomonas putida*) camphor hydroxylase system (23, 29). Because of their importance in xenobiotic and drug metabolism, these enzymes have also been thoroughly studied in mammalian liver (3, 5, 31). In contrast, Cyt P-450 of higher plant origin has not been the subject of extensive biochemical characterization, primarily because of the low content in many plant tissues, difficulties with identification in Chl containing tissues, and supposed lability in homogenized plant extracts.

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Some plant tissues are particularly enriched in spectrally detectable Cyt P-450 (6, 10, 12, 13, 19, 20). Cyt P-450 has been purified from tulip (*Tulipa gesneriana*) bulbs (12), and from tubers of Jerusalem artichoke (*Helianthus tuberosus*) (10). Additionally, an NADPH:Cyt P-450 reductase has been purified from Jerusalem artichoke (2), and the related Cyt $b_5$ and NADH:Cyt $b_5$ reductase have been purified from *Pisum sativum* (16). Unfortunately, the tulip bulb Cyt P-450 has not been associated with any enzymic activity, and polyclonal antibodies raised against it were not cross reactive with proteins from other species (11). The Jerusalem artichoke preparation can be reconstituted into its *trans*-cinnamate hydroxylase activity, but it has a low heme specific content and is not homogeneous (10). No primary sequence information has been reported for either of these proteins.

Reports of Cyt P-450 mediated xenobiotic metabolism in microsomes from the mesocarp of the California avocado (*Persea americana*) (6, 19) suggested that this fruit would be an appropriate high yielding source of a plant xenobiotic metabolizing Cyt P-450. We have purified from this tissue an active Cyt P-450 preparation, consisting of a mixture of two nearly identical polypeptides. Details of the avocado mesocarp Cyt P-450 structure and function are compared with its well characterized mammalian and bacterial counterparts.

## MATERIALS AND METHODS

Avocados (cv. Hass) were purchased locally, and ripened at room temperature. All HPLC was carried out at room temperature on a system previously described (21).

### Preparation of the Microsomal Fraction

Unless otherwise noted, all procedures are carried out on ice. Mesocarp tissue (typically ~150 g) was homogenized for 30 s in a blender with a buffer (2 mL/g of mesocarp) containing 0.1 M Mops-NaOH (pH 7.0), 0.3 M sorbitol, 5 mM EDTA, 0.1% (w/v) BSA, and 0.05% cysteine. The homogenate was centrifuged (30 min, 20,000*g*) affording three layers: a pellet, a supernatant, and an upper lipid layer. The supernatant was recovered, and recentrifuged (1 h, 100,000*g*). The pellet, defined as the microsomal fraction, was resuspended in ~1 mL of 0.1 M Mops-NaOH (pH 7.0), 50% (v/v) glycerol. It could be stored several months under liquid $N_2$.

### Solubilization of Microsomal Proteins

The resuspended microsomal fraction (~7 μM Cyt P-450, and ~20 mg/mL protein by the Bio-Rad assay) was diluted

with an equal volume of 2% (w/v), reduced Triton X-100 (RTX-100, Aldrich), 0.1M Mops-NaOH (pH 7.0) and was stirred for 15 min. After an addition of 1 M $MgCl_2$ (50 mM final), the mixture was recentrifuged (1 h, 100,000*g*). Solubilized Cyt P-450 was collected in the supernatant and could be stored several months at −80°C.

The efficiency of solubilization of the Cyt P-450, based on the concentration of Cyt in this supernatant, was typically 105 to 115% relative to microsomes; there was no detectable Cyt P-450 in the pellet. This apparent increase in Cyt P-450 concentration may reflect absorption flattening of the spectrum in the microsomal membrane (7) and/or the presence of a significant volume of particulate matter in the microsomal fraction. Unfortunately, it was difficult to remove the supernatant cleanly from the debris after centrifugation, and so net *recovery* of solubilized Cyt was often in the 50% to 60% range (see Table I).

## Preparation of Purified Native Avocado Cyt P450

The solubilized microsomal proteins were desalted at room temperature by passage over Sephadex G-25 in 20% (v/v)

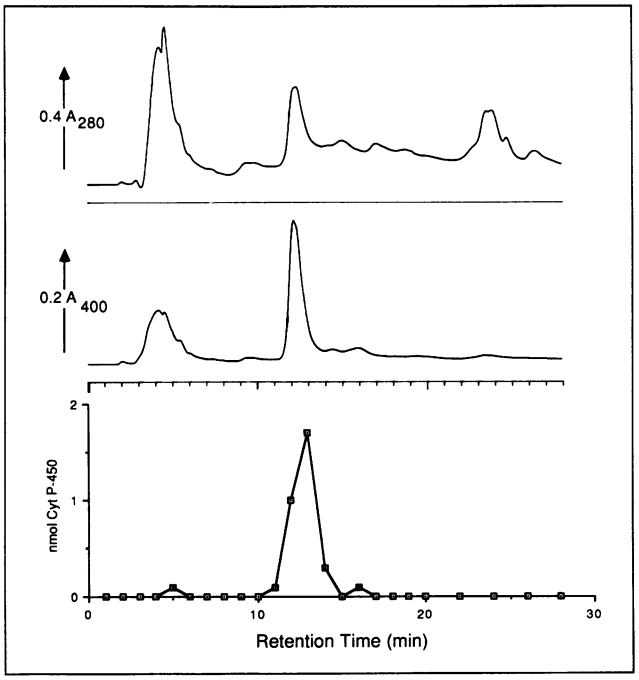


**Figure 1.** Anion exchange HPLC of solubilized avocado microsomal proteins. Solubilized proteins (1 mL at 9.1 μM Cyt P-450) were injected onto an LKB Instruments TSK-DEAE-5PW column (8 × 75 mm). Elution (flow = 0.75 mL/min) employed a series of linear gradients: starting with 100% buffer A, progressing from 0% to 50% buffer B between 1 and 20 min, and from 50% to 100% buffer B between 20 and 25 min. Buffer A was 20% (v/v) glycerol, 1% (w/v) RTX-100, 20 mM Tris-acetate (pH 7.0). Buffer B was buffer A supplemented with 0.8 M sodium acetate. The traces are (from top): $A_{280}$ (mostly general protein); $A_{400}$ (heme and/or other chromophores); P-450, quantitated as described in "Materials and Methods."

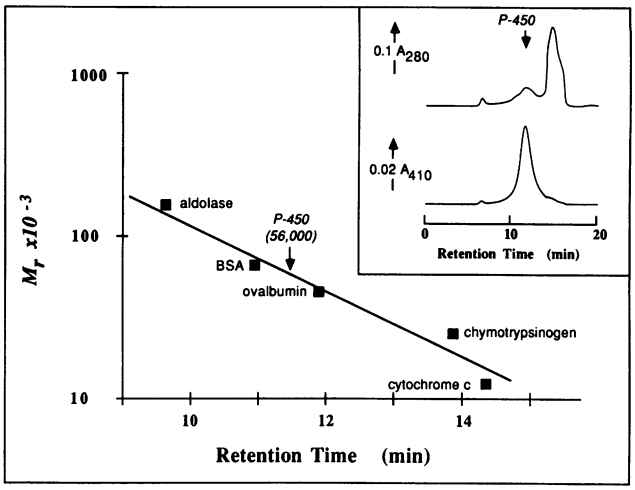


**Figure 2.** Gel filtration HPLC of the anion exchange purified cytochrome P-450. Avocado Cyt P-450 (~1 nmol) or mol wt standards were eluted from the column (LKB Instruments TSK G-3000SW, 7.5 × 300 mm, flow = 0.75 mL/min) in 0.1 M sodium phosphate buffer (pH 7.0), 0.1 M $Na_2SO_4$, 0.8% (w/v) CHAPS. Inset shows chromatograms of the avocado preparation at two detection wavelengths.

**Table I.** *Purification of Native Avocado Cyt P-450*

The yields from this procedure are based on a preparation from a single fruit providing 176 g of mesocarp tissue.

| Step | Cyt P-450 | Specific Content | Recovery |
|---|---|---|---|
| | *nmol* | *nmol/mg* | % |
| Microsomes | 22.9 | 0.3 | 100 |
| RTX-100 solubilization | 12.5 | 1.9 | 55 |
| Anion exchange chromatography (1% RTX-100) | 2.3 | ND[a] | 10 |
| Gel filtration chromatography (0.8% CHAPS) | 2.0 | 17.5 | 9 |

[a] ND = determined, because RTX-100 carryover from the ammonium sulfate concentration step made reliable protein measurement impossible.

glycerol, 1% (w/v) RTX-100,[2] 50 mM Mops-NaOH (pH 7.0), and were subjected to anion exchange HPLC as described in Figure 1. For preparative purposes, the Cyt P-450 was collected when the $A_{400nm}$ exceeded 0.5 $A_{280nm}$. This Cyt was precipitated with $(NH_4)_2SO_4$ (~0.4 g/mL) and collected by centrifugation (10 min, 10,000*g*). The floating precipitate was dissolved in 1% RTX-100, 50 mM Mops-NaOH (pH 7.0). This protein could be further treated to exchange RTX-100 for CHAPS (Sigma), by gel filtration HPLC as described in Figure 2. The Cyt P-450 containing fraction was collected when $A_{420} \geq 0.8\ A_{280}$.

## Gel Electrophoresis and Immunoblot

The use of LDS PAGE performed at 4°C enabled optimal separation of membrane proteins (17). In some cases long (30

[2] Abbreviations: RTX-100, reduced Triton X-100; CHAPS, 3-([3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate; LDS, lithium dodecyl sulfate; PCMA, *p*-chloro-*N*-methylaniline; TFA, trifluoroacetic acid.

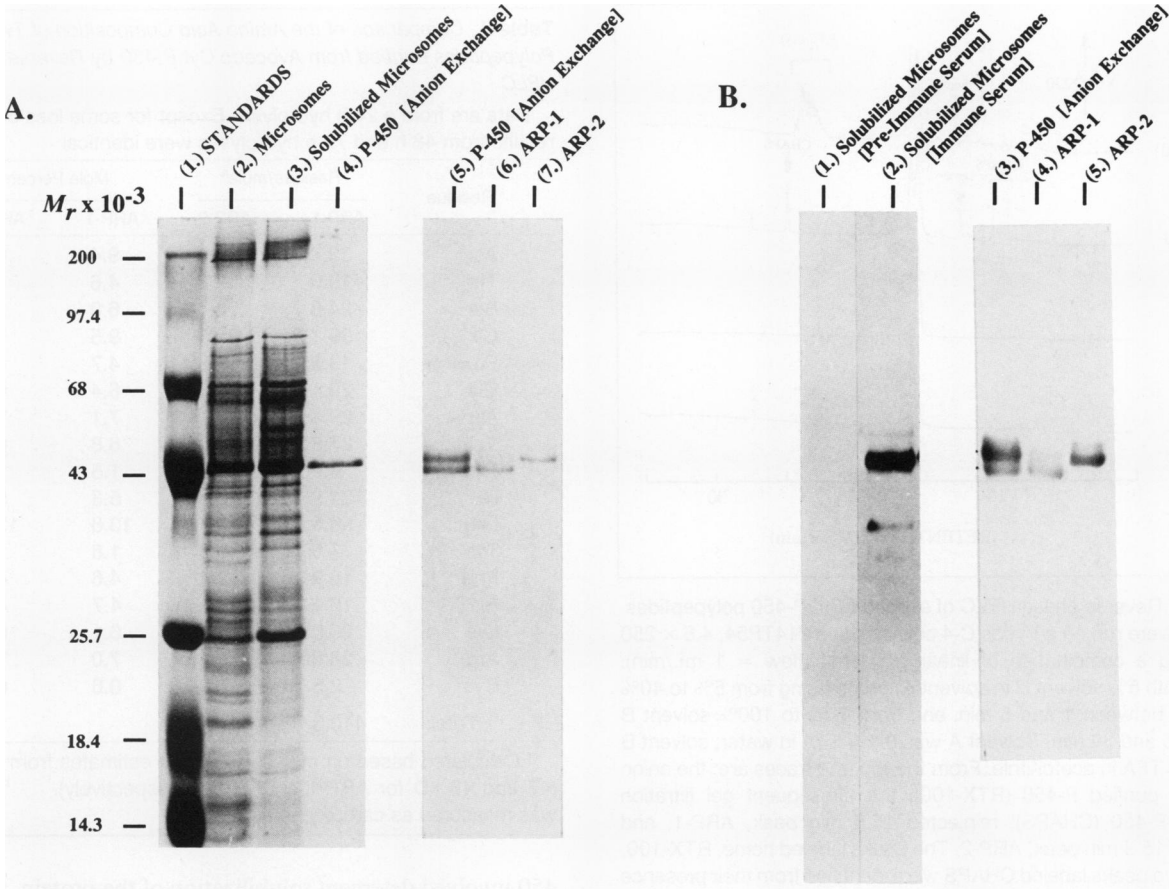


**Figure 3.** LDS gel electrophoresis of avocado Cyt P-450. A, Coomassie stained gel, run either in a 10% to 17% polyacrylamide gradient (lanes 1–4), or in a homogenous 10% polyacrylamide gel (lanes 5–7). The amounts of proteins loaded on the gel were: ~150 μg (lanes 2 and 3), ~5 μg (lanes 4 and 5), ~1 μg (lanes 6 and 7). B, Western blot using either preimmune serum (lane 1), or anti-ARP-1 serum (lanes 2–5). The proteins were separated either on a gradient as in A (lanes 1 and 2), or a 10% homogenous gel (lanes 3–5). The amounts of proteins loaded on this gel were: ~150 μg (lanes 1 and 2), ~5 μg (lane 3), ~1 μg (lanes 4 and 5).

cm) gels were employed to increase the resolution of tightly spaced bands (21). Inclusion of DTT in the upper buffer chamber (1 mM) and sample buffer (50 mM) significantly increased the resolution of microsomal proteins. Myosin (200,000), phosphorylase B (97,400), BSA (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), β-lactoglobulin (18,400), and lysozyme (14,300) were used as mol wt standards.

Polyclonal antibodies (Hazleton Research Products, Denver, PA) were elicited in New Zealand White rabbits by two subcutaneous injections of highly purified Cyt P-450 polypeptide, ARP-1 (125 μg protein primary injection, in Freund's complete adjuvant, followed after 2 weeks by 125 μg in Freund's incomplete adjuvant). The ARP-1 had been collected from reverse phase HPLC of anion exchange purified Cyt P-450, and evaporated to near dryness to remove acetonitrile and TFA.

For Western blots, proteins were electrophoretically transferred from acrylamide gels to nitrocellulose (80 V overnight, 4°C) according to Towbin *et al.* (30) except that transfers were done in the absence of detergent. With the exception that 30 mg/mL BSA (1 h) blocking solution was used, the blot was developed (1:1000 dilution of ARP-1 antiserum for 2 h, 1:2000 dilution goat-anti-rabbit alkaline phosphatase conjugate for 2 h) using the Bio-Rad Immun-Blot assay kit.

### Protein Analyses

Absorption spectra of microsomes were measured on a Johnson Foundation SDB-3A scanning dual wavelength spectrophotometer. Spectra of the purified proteins were measured on a Hewlett-Packard model 8450A diode array spectrophotometer. Cyt P-450 concentration was estimated from the absorption difference spectrum of the ferrous:CO complex *versus* the ferrous Cyt (22).

Routine microsomal protein amounts were estimated by the Bio-Rad protein assay, using BSA as standard. This technique gave values greater than twice those obtained by the Lowry procedure (18). Neither the Bio-Rad nor the Lowry procedure could be used directly for RTX-100 or CHAPS solubilized preparations. However, CHAPS (≤0.8%) or RTX-100 (≤1%) interference was eliminated if the proteins were precipitated with an equal volume of 20% TCA, centrifuged, washed twice with ethanol, redissolved in 0.1 M NaOH, and analyzed by the Lowry protein determination.

Heme content of the purified Cyt was determined by the method of Appleby (1).

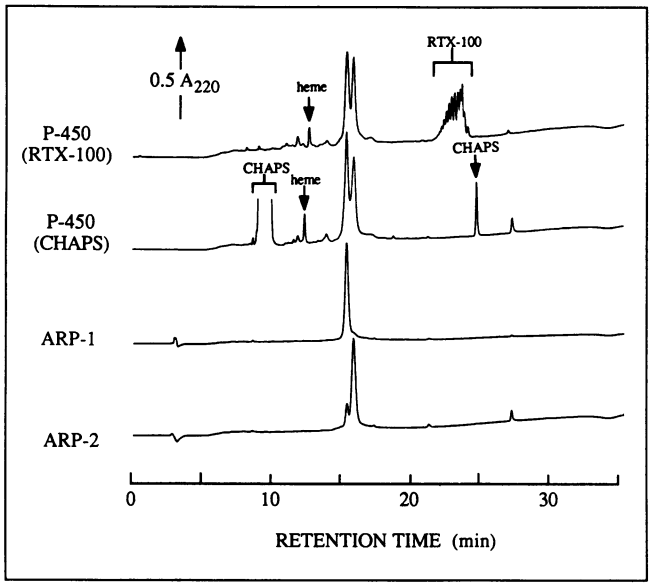


**Figure 4.** Reverse phase HPLC of avocado Cyt P-450 polypeptides. Samples were run on a Vydac C-4 column (type 214TP54, 4.6 × 250 mm) using a combination of linear gradients (flow = 1 mL/min): starting with 5% solvent B in solvent A, progressing from 5% to 40% solvent B between 1 and 5 min, and from 40% to 100% solvent B between 5 and 30 min. Solvent A was 0.1% TFA in water; solvent B was 0.1% TFA in acetonitrile. From the top, the traces are: the anion exchange purified P-450 (RTX-100); the subsequent gel filtration collected P-450 (CHAPS); reinjected 15.3 min peak, ARP-1; and reinjected 15.9 min peak, ARP-2. The peaks labeled heme, RTX-100, and the two peaks labeled CHAPS were identified from their presence at identical retention times in standards.

### Amino Acid Analysis and N-Terminal Sequencing

All analyses were conducted on samples purified by reverse phase HPLC. Samples for amino acid analysis were hydrolyzed in 6 M HCl at 110°C for 24, 48, and 72 h. To quantitate cysteine, samples were reduced with DTT, alkylated with iodoacetic acid, and rechromatographed (26). Amino acid analysis was performed on a Beckman 6300 ion exchange/ninhydrin analyzer. N-terminal sequencing was done by automated Edman degradation (26).

### Enzymatic Activity

PCMA demethylase activity was assayed fluorimetrically, according to Rifkind and Petschke (24). For the cumene hydroperoxide dependent demethylase measurements, samples (0.5 mL) contained ~250 nM Cyt P-450, 0.8 mM PCMA (HCl salt, Calbiochem), and 0.1 M Hepes-NaOH (pH 7.0); solubilized preparations were supplemented with 0.8% CHAPS. To initiate the reaction, 2 μL of cumene hydroperoxide (~6 mM final) was added with rapid stirring. The reaction was incubated at room temperature, terminated by the addition of 0.1 mM 30% trichloroacetic acid, and analyzed for *p*-chloroaniline content (24).

## RESULTS

### Preparation of a Purified Cyt P-450

After preparation of a crude microsomal fraction from avocado mesocarp, the next step in purification of the Cyt P-450 involved detergent solubilization of the protein. Effective solubilization of the membrane bound cytochrome could be achieved with a number of detergents already used for this purpose: Igepal CO-710 (GAF Corp., equivalent to Emulgen 911), CHAPS, and Triton X-100 (10, 12, 20). However, reduced Triton X-100 (RTX-100) was effective and most suitable for solubilization prior to purification because of its compatibility with anion exchange chromatography and its low uv absorbance at 280 nm. Figure 1 shows the results of anion exchange chromatography (on TSK-DEAE 5PW) of the solubilized microsomes; the Cyt P-450 was both the major chromophore and a major protein component of the mixture. Although not shown, the Cyt P-450 also eluted as a single peak from mono-Q anion exchanger (Pharmacia), and high resolution spherical hydroxylapatite (Regis).

Although gel filtration chromatography (Fig. 2) afforded little further purification of the Cyt (see Fig. 4 and Table I), this step did remove residual RTX-100 from the Cyt when the zwitterionic detergent CHAPS was included in the elution buffer (note the RTX-100 peak at ~14 min in the upper trace of Fig. 2 inset). Many of the experiments described in this text have been performed both on the anion exchange purified Cyt P-450 containing RTX-100, and on the same protein with CHAPS subsequently replacing RTX-100 by means of this gel filtration step. The yields and specific contents of these two native preparations in a typical purification are shown in Table I.

**Table II.** *Comparison of the Amino Acid Composition of Two Polypeptides Purified from Avocado Cyt P-450 by Reverse Phase HPLC*

Data are from a 24 h hydrolysis. Except for some loss of histidine, results from 48 h and 72 h hydrolyses were identical

| Residue | Residue/mole[a] | | Mole Percent | |
|---|---|---|---|---|
| | ARP-1 | ARP-2 | ARP-1 | ARP-2 |
| Asx | 38.7 | 38.6 | 9.4 | 9.2 |
| Thr | 19.0 | 19.2 | 4.6 | 4.6 |
| Ser | 24.6 | 24.4 | 6.0 | 5.8 |
| Glx | 39.1 | 39.6 | 9.5 | 9.5 |
| Pro | 19.2 | 19.3 | 4.7 | 4.6 |
| Gly | 26.4 | 26.8 | 6.4 | 6.4 |
| Ala | 29.3 | 30.1 | 7.1 | 7.2 |
| Val | 27.8 | 29.0 | 6.8 | 6.9 |
| Met | 6.2 | 6.7 | 1.5 | 1.6 |
| Ile | 21.9 | 22.6 | 5.3 | 5.4 |
| Leu | 56.5 | 57.7 | 13.8 | 13.8 |
| Tyr | 7.5 | 7.6 | 1.8 | 1.8 |
| Phe | 18.9 | 19.4 | 4.6 | 4.6 |
| His | 19.1 | 20.1 | 4.7 | 4.8 |
| Lys | 25.0 | 25.4 | 6.1 | 6.1 |
| Arg | 28.6 | 29.3 | 7.0 | 7.0 |
| Cys[b] | 2.5 | 2.8 | 0.6 | 0.7 |
| Total: | 410.3 | 418.4 | | |

[a] Calculated based on molecular weight estimates from LDS gels (47 and 48 kD for ARP-1 and ARP-2, respectively). [b] Cysteine was measured as carboxymethylcysteine.

### Properties of Denatured Avocado Cyt P-450

The composition of the anion exchange purified preparation was investigated further by LDS gel electrophoresis and

**Table III.** *N-Terminal Sequencing of Two Polypeptides Purified from Avocado Cyt P-450 by Reverse Phase HPLC*

| Cycle | ARP1 (392 pmol) | | ARP2 (218 pmol) | |
|---|---|---|---|---|
| | Residue | pmol[a] | Residue | pmol[a] |
| 1 | Ala | 172 | Met | 106 |
| 2 | Ile | 118 | Ala | 78 |
| 3 | Leu | 150 | Ile | 76 |
| 4 | Val | 106 | Leu | 83 |
| 5 | Ser | 40 | Val | 67 |
| 6 | Leu | 106 | Ser | 31 |
| 7 | Leu | 112 | Leu | 65 |
| 8 | Phe | 88 | Leu | 61 |
| 9 | Leu | 108 | Phe | 60 |
| 10 | Ala | 104 | Leu | 62 |
| 11 | Ile | 60 | Ala | 64 |
| 12 | Ala | 99 | Ile | 43 |
| 13 | Leu | 101 | Ala | 58 |
| 14 | Thr | 32 | Leu | 54 |
| 15 | Phe | 66 | Thr | 23 |
| 16 | Phe | 72 | Phe | 47 |
| 17 | Leu | 90 | Phe | 43 |
| 18 | Leu | 100 | Leu | 45 |
| 19 | Lys | 18 | Leu | 52 |
| 20 | Leu | 82 | Lys | 17 |
| 21 | Asn | 33 | Leu | 41 |
| 22 | Glu | 42 | Asn | 20 |
| 23 | Lys | 16 | Glu | 28 |
| 24 | Arg | 43 | Lys | 17 |
| 25 | Glu | 41 | Arg | 22 |
| 26 | Lys | 13 | Glu | 26 |
| 27 | Lys | 16 | Lys | 14 |
| 28 | Pro | 37 | Lys | 15 |
| 29 | Asn | 28 | Pro | 23 |
| 30 | Leu | 64 | Asn | 15 |
| 31 | Pro | 43 | Leu | 37 |
| 32 | Pro | 46 | Pro | 23 |
| 33 | X | | Pro | 25 |
| 34 | Pro | 35 | Ser | 8 |
| 35 | | | Pro | 21 |
| 36 | | | Pro | 20 |
| 37 | | | Asn | 13 |
| 38 | | | Leu | 31 |
| 39 | | | Pro | 17 |
| 40 | | | Ile | 19 |

[a] Amounts on each cycle are not corrected for carryover from the previous cycle.

reverse phase HPLC, both performed under denaturing conditions. Figure 3A, lanes 2 to 4, shows the results of LDS gel electrophoresis of avocado microsomes, RTX-100 solubilized microsomes, and anion exchange purified Cyt P-450. This survey gel indicates that the anion exchange preparation contains a major 47 kD polypeptide and is free from other major impurities. However when the 40 to 50 kD region of the gel was expanded on a long homogeneous 10% gel, this region obviously contained a doublet of polypeptides at about 47 and 48 kD (Fig. 3A, lane 5). This heterogeneity was confirmed by reverse phase HPLC (Fig. 4, peaks at 15.3 and 15.9 min). LDS gel electrophoresis of the earlier (ARP-1) and later (ARP-2) reverse phase peaks identified them as the 47 and 48 kD polypeptides, respectively (Fig. 3A, lanes 5 to 7).

ARP-1 was used to elicit a polyclonal antibody, which cross reacted with ARP-1 and somewhat more strongly to ARP-2 (Fig. 3B). In addition to the major band attributable to overlapping ARP-1 and ARP-2 polypeptides, solubilized microsomes (Fig. 3B, lane 2) also contain two minor antigenic bands at lower molecular weight (possibly proteolytic fragments), and one minor band at higher mol wt, the significance of which is unclear.

The chromatographic, electrophoretic, and immunological similarities of ARP-1 and ARP-2 are reflected in their amino acid compositions and N-terminal sequences. The amino acid compositions of ARP-1 and ARP-2 are shown in Table II. While the residue/mole comparison shows some minor differences, ARP-1 and ARP-2 are virtually indistinguishable on a mole percent basis. N-terminal sequencing (Table III) gave identical sequences for 34 residues, except that an N-terminal methionine only on ARP-2 makes its sequence one residue out of phase with ARP-1. Furthermore, reverse phase analyses of tryptic digests of ARP-1 and ARP-2 (Fig. 5) were nearly identical. There are four notable differences; of these, the peak at ~6.2 min in ARP-1 is minor, and that at ~15.6 min in ARP-2 may not be a peptide, as judged by its atypical absorption spectrum and amino acid analysis. Those at ~26.5 and ~31.6 min in the ARP-1 digest may represent significant differences. Nonetheless, if we assume that identical retention times indicate identical polypeptides, integration of the peak area of these peptides in Figure 5 suggests that ARP-1 and ARP-2 are composed of a minimum of 92% identical fragments.

Both ARP-1 and ARP-2 polypeptides were present in preparations from a single fruit. Amounts and relative proportions were unaffected by protease inhibitors (0.5 mM PMSF and 1 mg/L leupeptin) present from homogenization to immediately prior to anion exchange chromatography. These findings suggest that both polypeptides occur *in situ*, and that neither is a proteolytic artifact of the isolation procedure. Western blot analysis of both avocado leaf and root microsomal fractions with the ARP-1 antibody revealed no cross reacting proteins (data not shown), suggesting that antigenically related Cyt P-450 are not widely distributed throughout the plant.

### Properties of Native Avocado Cyt P-450

Gel filtration of ion exchange purified Cyt P-450 in either RTX-100 (not shown) or CHAPS (Fig. 2) gave an apparent mol wt of ~56,000; comparison of this value to the estimate of ~47,000 for the apoprotein obtained by LDS gel electrophoresis suggests that the purified protein is monomeric. The chromophore of the enzyme was tentatively identified as ferriprotoporphyrin IX by both pyridine hemochromogen assay (1) and retention time on reverse phase HPLC (Fig. 4) compared to authentic heme protein standards (myoglobin, catalase). The specific content of heme from the pyridine hemochromogen assay (15.9 nmol/mg protein), or from the extinction coefficient (22) for Cyt P-450 chromophores (17.9 nmol/mg protein; see Table I), are close to the expected value of 21 nmol/mg for a 47 kD protein with one heme per monomer. Further, these values are high enough to make it unlikely that the native form of either ARP-1 or ARP-2 is a chromophore free protein, although we cannot rule out the

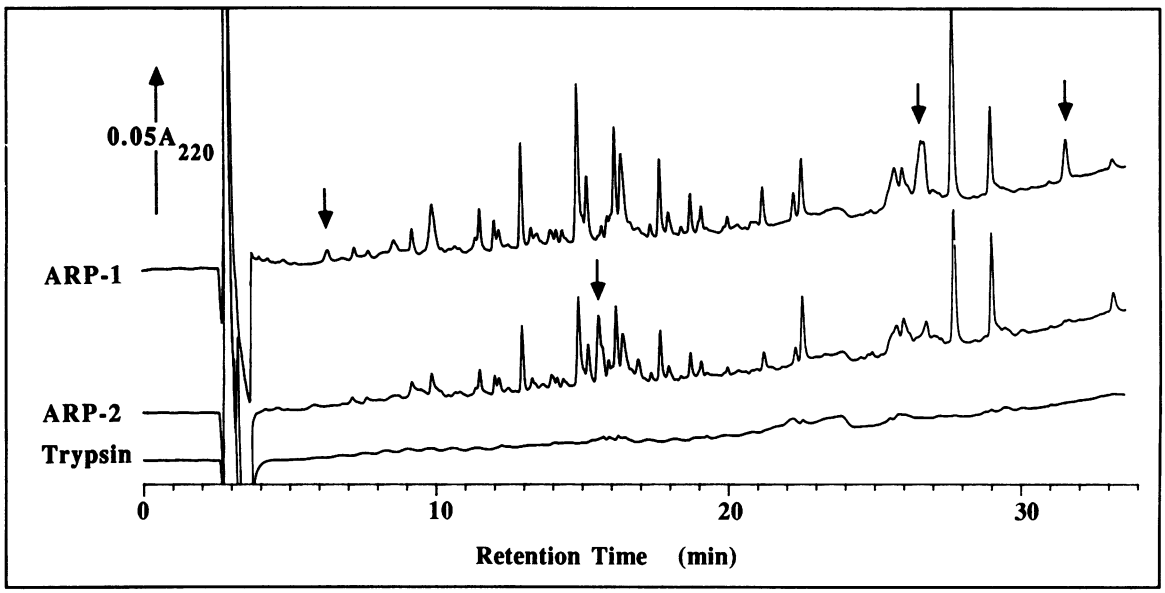


**Figure 5.** Reverse phase HPLC of ARP-1 and ARP-2 trypsin digests. Samples corresponding to ~1 nmol of each polypeptide, purified by reverse phase HPLC as in Figure 4, were evaporated to near dryness, redissolved in 0.2 M Tris-HCl (pH 8.5), and incubated for 20 h total at 37°C with 1 μg *N*-tosyl-L-phenylalanylchloromethyl ketone (TPCK)-trypsin (Worthington) added initially and again at 15 h. The digestion was stopped by freezing, and the digests were analyzed on a C-4 reverse phase column. Chromatographic conditions were the same as in Figure 4 except the gradient started at 5% solvent B, progressed from 5% to 70% solvent B between 1 and 30 min, and from 70% to 100% solvent B between 30 and 31 min. The bottom trace is from a TPCK-trypsin incubation with no additional protein.

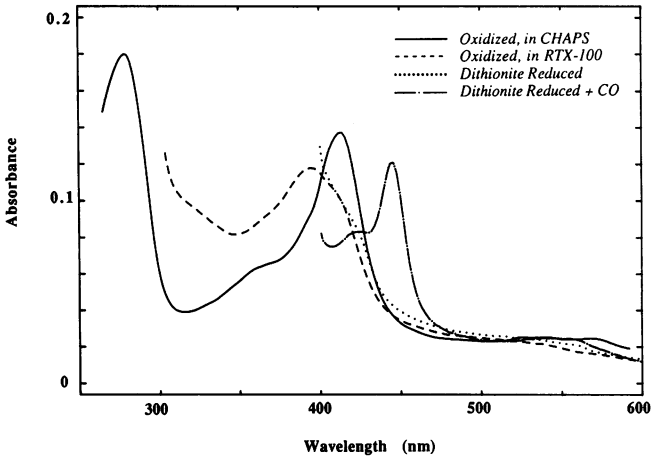


**Figure 6.** Absorption spectra of purified native avocado Cyt P-450. Both the CHAPS and RTX-100 containing proteins were diluted into 0.1 M Mops-NaOH (pH 7.0), 0.8% CHAPS. The other spectra are from the addition to CHAPS containing protein of a few crystals of solid sodium dithionite and subsequently bubbling for 30 s with CO.

unprecedented possibility that one of them contains 2 hemes/polypeptide.

Replacement of RTX-100 by CHAPS is accompanied by a marked change in the spectroscopic properties of the protein (Fig. 6). In the anion exchange purified protein still containing RTX-100, the Soret (gamma) band is at ~390 nm, which is characteristic of a predominantly high spin (S = 5/2) ferricytochrome P-450. After replacement of the RTX-100 with CHAPS, the Soret band was at ~418 nm, typical of a low spin (S = 1/2) ferricytochrome P-450 (15). This spectral shift could be reversed by subsequent gel filtration in RTX-100. These data suggest that surfactant binding can affect the spin state of the Cyt P-450. No data on the spectral or catalytic properties of the protein in the absence of detergent could be obtained, since dilution of the detergent or gel filtration in the absence of any detergent resulted in the aggregation and precipitation of the cytochrome. Figure 6 also shows the changes in the absorption spectrum of the CHAPS containing protein upon reduction with dithionite, and upon formation of the ferrocytochrome:CO complex, with its characteristic maximum at ~450 nm.

### Substrate Binding and Enzymatic Activity

The avocado Cyt P-450 was first described as a microsomal PCMA demethylase, though its physiological substrates were not identified (6). Difficulties inherent in measuring the Soret maximum of the Cyt in a highly scattering microsomal suspension preclude direct measurement of the absorption maximum and interpretation of the spin state of the membrane bound enzyme. However, we have found by difference spectroscopy that PCMA binding to the membrane bound cytochrome P-450 produces a typical "type I" spectral shift (Fig. 7A), indicating that binding is accompanied by a shift in the heme spin equilibrium toward the high spin state. Figure 7D (filled circles) shows that the apparent $K_D$ for PCMA in effecting this change is 180 μM. Upon addition of saturating (0.2 mM) NADPH, turnover occurs with a $K_M$ of 200 μM for PCMA and a $V_{max}$ of 8 $min^{-1}$ (calculated from Ref. 6; confirmatory data not shown).

In contrast to the microsomal protein, the unusual difference spectrum from the RTX-100 containing enzyme most closely resembles a 'reverse type I' spectrum (15) indicating a

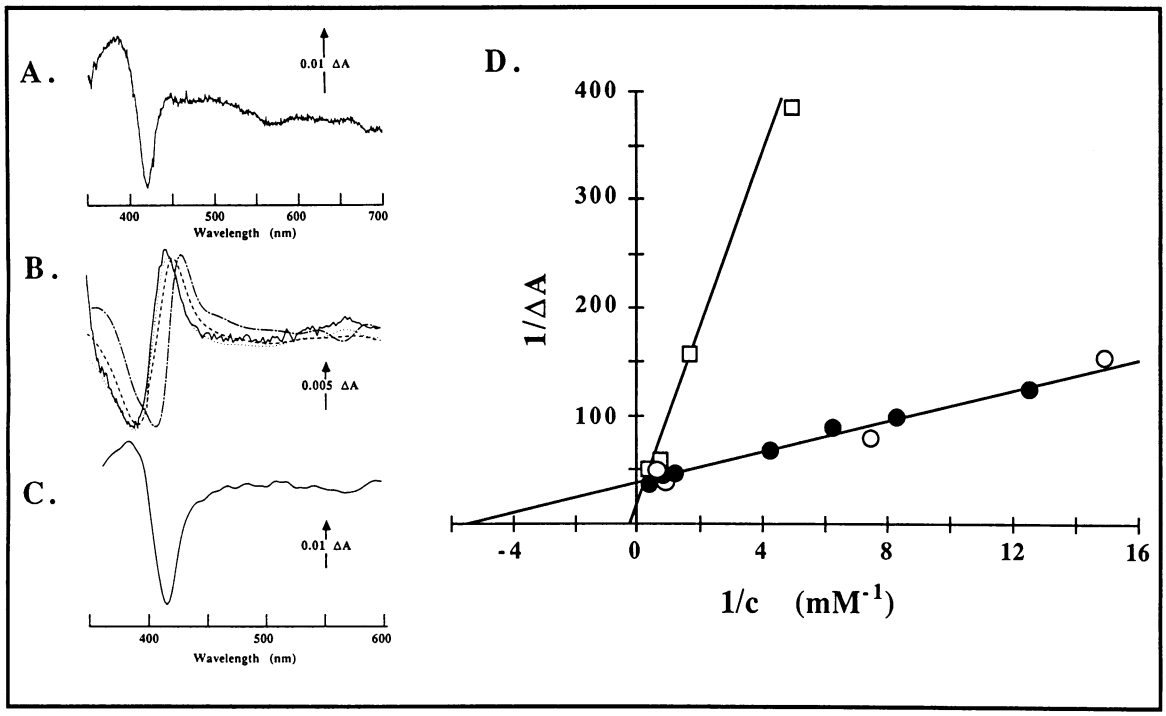


**Figure 7.** PCMA induced difference spectra of avocado Cyt P-450. The Cyt P-450 containing samples were diluted to ~1 μM, and the difference spectra show the absorption changes obtained upon the addition of PCMA. A, Avocado microsomal fraction in 0.1 M Mops-NaOH (pH 7.0), 5 mM EDTA. The difference spectrum from the addition of 0.8 mM PCMA was measured by dual wavelength spectroscopy. B, Anion exchange purified Cyt P-450 in 0.1 M Mops-NaOH (pH 7.0), 1% RTX-100, difference spectrum from the addition of 1.3 mM PCMA (solid line). For comparison, the lineshape of a type IIA spectrum (dashed line), a type IIB spectrum (dash-dot), and a reverse type I spectrum (dotted line) are shown (15, 21, 27). C, Purified Cyt P-450 which has had RTX-100 exchanged for CHAPS; in 0.1 M Mops-NaOH (pH 7.0), 0.8% CHAPS. Difference spectrum is from the addition of 1.3 mM PCMA. D, Double reciprocal plot of the concentration dependence of the PCMA induced difference spectra shown in A (filled circles), B (open squares), and C (open circles). For A and C, $\Delta A$ refers to the absorption change at 390 – 419 nm; for B, at 418 – 390 nm.

**Table IV.** *Cumene Hydroperoxide Dependent Demethylation of PCMA*

Rates were measured over a 3 min incubation. The assay was shown to be reasonably linear over 5 min, although cumene hydroperoxide did inactivate the enzyme.

| Sample | Activity |
|---|---|
| | *nmol PCA·[nmol P-450]$^{-1}$·min$^{-1}$* |
| Microsomes | 85 |
| P-450 (anion exchange) | 31 |
| P-450 (gel filtration) | 42 |

transition from high to low spin upon substrate addition (Fig. 7B). The titration curve (Fig. 7D, open squares) suggests both that the maximum extent of the absorption change more than doubles and that the apparent $K_D$ for PCMA increases to ~4 mM. However, when RTX-100 is replaced with CHAPS (Fig. 7, C and D, open circles), substrate binding parameters are indistinguishable from those of the microsomal protein. Taken together, these data suggest that the protein in CHAPS closely resembles the native microsomal enzyme. Furthermore, the similarities in substrate binding suggest that, like the isolated protein in CHAPS, the microsomal Cyt is predominantly low spin.

Physiological turnover of Cyt P-450 dependent enzymes requires the presence of reduced pyridine nucleotide, molecular oxygen, and a reductase system to transfer reducing equivalents from NAD(P)H to the Cyt. *In vitro* it is often possible to replace these accessory components with an organic peroxide, such as cumene hydroperoxide, which provides both reducing power and an oxygen source (5, 29). Table IV shows that cumene hydroperoxide supported PCMA demethylase activity of a microsomal preparation, and the RTX-100 or CHAPS containing purified avocado enzymes. These purified fractions retained substantial activity, even though no attempt was made to optimize assay conditions. Turnover in microsomes with cumene hydroperoxide (85 min$^{-1}$) was about 10 times faster than with NADPH, suggesting that electron transfer in the NADPH supported reaction may be severely rate limiting. Addition of PCMA and NADPH to the purified Cyt P-450 in CHAPS or RTX-100 did not result in detectable PCA production, presumably because purification has removed the reductase system. Addition of 0.5 U (1 U = reduction of 1 μmol Cyt *c*/min) rabbit liver NADPH:Cyt P-450 reductase to RTX-100 containing Cyt P-450, plus PCMA and NADPH, resulted in slight but measurable activity, with a turnover of ~0.1 min$^{-1}$.

## DISCUSSION

The data presented here demonstrate that it is possible to purify an enzymically active Cyt P-450 protein from the

microsomal fraction of the avocado fruit mesocarp. Denaturing high resolution separation techniques show that this protein is clearly a mixture of two polypeptides, ARP-1 and ARP-2. While it is a consideration that the native 'purified' cytochrome is not homogeneous, the stoichiometry of heme to protein (Table I) suggests that the native preparation is entirely composed of Cyt P-450 protein. Both the substrate binding studies (Fig. 7) and kinetic analyses of PCMA demethylase activity (6) (confirmed in our laboratory) provide no evidence that avocado mesocarp microsomal Cyt P-450 contains more than one functional population of monooxygenase. This suggests either that the native forms of ARP-1 and ARP-2 interact similarly with PCMA, or that one interacts much more strongly than the other. Studies with other substrates could clarify the functional relationship between the two proteins.

We are presently unable to define the precise relationship between ARP-1 and ARP-2. The difficulty in separating the proteins suggests that they are very similar. They were not resolved by high performance anion exchange chromatography, although this technique can resolve a variety of soluble (20, 21) and membrane bound (9, 20) Cyt P-450 in extracts from other organisms. ARP-1 and ARP-2 can be separated by reverse phase HPLC, which is capable of resolving smaller proteins differing by as little as a single amino acid (14, 25). The cross-reactivity of both ARP-1 and ARP-2 with the ARP-1 antibody, as well as the similarities of their amino acid analyses, N-terminal sequences, and tryptic digestion patterns, all suggest that major portions of these two polypeptides are identical. However, in the absence of complete sequence information, we cannot tell if they originate from a single gene product which undergoes post-translational modification(s), or from separate genes. This first possibility is consistent with what we know to be the minimal difference between ARP-1 and ARP-2 and would require removal of the N-terminal methionine from some ARP-2 to yield a mixture of ARP-1 and ARP-2. A second possibility is reminiscent of the situation with some structurally similar Cyt P-450 which have been found to be distinct gene products. Rat liver Cyt P-450b and P-450e are such a case. These two proteins share 478 of 491 residues, cross-react with the same antibodies, and give similar tryptic digests (3, 9, 31). In spite of these similarities, native Cyt P-450b and P-450e can be resolved by anion exchange HPLC under conditions nearly identical to those used here (9). Notably, while the apparent mol wt difference in these two proteins on SDS gels is ~700 (3, 31), the difference calculated from their primary sequence (3) is only 17!

The N-terminal sequences of ARP-1 and ARP-2 bear two striking similarities to those of mammalian Cyt P-450. The first 18 or 19 residues of ARP-1 and ARP-2 are hydrophobic or only weakly polar (Table III), and 7 of the following 9 residues are charged (residues 20 to 28 in ARP-2 numbering). This pattern (a hydrophobic stretch long enough to be transmembrane, followed by a region of charged residues) is typical of a combined insertion-halt-transfer signal responsible for proper membrane insertion (3, 28). The second feature common to many Cyt P-450 is the presence of a 'proline cluster' of unknown function near the N terminus (3); between residues 29 and 39 of ARP-2, 6 of the 11 amino acids are proline.

While some data on purified higher plant Cyt P-450 are available in the literature (10, 12, 13), we believe ours is the first report to include primary sequence information or extensive characterization of more than a single polypeptide. Most Cyt P-450 containing plant tissues that have been analyzed contain one, or only a very few Cyt P-450 (10, 12, 20) (this work). Some of the results of this work, particularly the antibody and the N-terminal sequences, should provide useful tools for further assessing the presence of Cyt P-450 isozymes in other plant tissues.

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