Low Carbon Monoxide Affinity Allene Oxide Synthase Is the Predominant Cytochrome P450 in Many Plant Tissues

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ABSTRACT: A cytochrome P450 with low affinity (2.9×10^3 M^{-1}) for CO appears to be the major microsomal P450 in some plant tissues. The presence of low CO affinity cytochrome P450 correlates with its lack of NADPH reducibility and with the presence of high levels of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoate peroxidase activity. This activity and low CO affinity are retained by purified tulip cytochrome P450, which appears to be catalytically identical to a flaxseed-derived fatty acid allene oxide synthase P450 described previously [Song, W.-C., & Brash, A. R. (1991) *Science 253,* 781–784]. Other heme-binding ligands, such as CN^- and imidazoles, bind weakly to the allene oxide synthase P450s, suggesting that axial coordination in the heme distal pocket may be hindered. We conclude that low CO affinity is characteristic of the allene oxide synthase P450s and that these P450s constitute a major portion of the microsomal P450 in a variety of plant tissues, particularly from monocot species.

Cytochrome P450 enzymes have been identified in a number of plant species, several have been purified, and cDNA has been cloned and sequenced for one (Higashi et al., 1985; O'Keefe & Leto, 1989; Bozak et al., 1990; Gabriac et al., 1991; Kochs et al., 1992). These enzymes are known to be involved in a number of biosynthetic pathways leading to the formation of phenylpropanoids, monoterpenes, sterols, and gibberellins. Many xenobiotics are also metabolized in P450[1] dependent reactions, particularly in monocots (Frear et al., 1991). In animal liver, xenobiotics are metabolized by a group of enzymatically nonspecific P450 monooxygenase enzymes, utilizing molecular oxygen, and reducing equivalents from NADPH provided via an NADPH:P450 oxidoreductase enzyme. It is not clear whether individual plant species have as many distinct multifunctional P450s as are found in typical animal liver. Molecular approaches using probes derived from plant P450 sequences are useful in defining the variety of plant P450s, especially when used in combination with yeast expression systems to determine their function (Bozak et al., 1992; O'Keefe et al., 1992). However, functional attributes, particularly substrate specificity and heme ligand binding properties, may provide a faster and more general means to categorize and distinguish individual forms among the mixture of total microsomal P450.

Recently, the purification of a cytochrome P450 from flaxseed, identified as an allene oxide synthase (AOS), was reported (Song & Brash, 1991). This enzyme appears to be part of a metabolic pathway (also including lipoxygenase) which produces the plant growth regulator jasmonic acid from unsaturated free fatty acids (Vick & Zimmerman, 1984). Unlike the reductant and O_2 requiring P450 monooxygenase, the AOS P450 is unique in that it utilizes a hydroperoxide substrate (i.e., oxygen that is 2 equiv reduced above O_2), so it requires neither O_2 or reductant. If this is the sole physiological function of this P450, it would not need to interact with NADPH:P450 oxidoreductase or to be able to coordinate O_2 to the ferrous heme.

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[1] Abbreviations: AOS, allene oxide synthase; 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; P450, cytochrome P450.

In the results reported here, we identify a P450 isolated from tulip bulb as an AOS. This protein has a decreased affinity (~60-fold) for the ferrocytochrome ligand CO when compared to other plant P450s which carry out monooxygenase reactions. Microsomal fractions from a variety of plant sources have been found to contain cytochromes P450 with low affinity for CO, and this property correlates with the presence of AOS activity, and the lack of NADPH reducibility of the cytochrome. These correlations suggest that both properties, the hindered binding of CO and lack of NADPH reducibility, are the consequence of unique structural features related to the mechanism of AOS catalysis. The properties may also be used diagnostically to differentiate between typical monooxygenase P450s and the AOS or related P450s in plant microsomal fractions.

EXPERIMENTAL PROCEDURES

Microsomal and Protein Preparations. Microsomal fractions were prepared and stored under liquid nitrogen as previously described (O'Keefe & Leto, 1989) from prevernalized tulip (*Tulipa gesneriana* cv. Red Apeldoorn) bulbs, ripe avocado (*Persea americana* cv. Hass) mesocarp, Jerusalem artichoke (*Helianthus tuberosus*) tubers homogenized directly (uninjured) or sliced 2-mm thick and aged 24 h in water at 28 °C, 4- or 5-day-old etiolated wheat (*Triticum aestivum* cv. Era) coleoptiles, and dry milled corn (*Zea mays*) germ (kindly provided by Mr. Harry Frost, Illinois Cereal Mills, Paris, IL).

Avocado P450 was purified as previously described (O'Keefe & Leto, 1989). Purified tulip P450 was prepared by a modification of a technique previously described (Higashi et al., 1985), substituting Triton X-100 for the detergent during solubilization (1.5% w/v final) and chromatography (0.2%). Other modifications include substituting a Mono-Q column for DEAE cellulose, and eliminating the second anion-exchange step. A final step in the purification was added, incorporating gel filtration chromatography on a TSK G3000SW column (7.5 × 300 mm, 0.75 mL/min) in a buffer consisting of 0.1 M sodium phosphate (pH 7.0), 0.2 M Na_2SO_4, and 1% (w/v) octyl-β-D-glucopyranoside. Nearly com-

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plete removal of Triton X-100 at this step facilitated subsequent concentration of the protein by ultrafiltration.

Antibodies were prepared at HRP Inc. (Denver, PA) by intranodal injection of 67 μg of purified tulip P450 in Freund's complete adjuvant into a New Zealand white rabbit. Subsequent injections of 67, 33, and 33 μg were made in Freund's incomplete adjuvant at 30-day intervals. Western blot analysis using the rabbit antiserum was carried out using goat antirabbit alkaline phosphatase conjugate and was developed using the BioRad Immuno-Blot assay kit.

CO Equilibrium Studies and Redox Titrations. CO equilibrium studies were carried out at room temperature in 0.1 M MOPS–NaOH (pH 7.0) using protein (purified or microsomal) corresponding to 0.2–0.5 μM cyt P450. A fresh protein sample was used for each concentration of CO. Protein samples were reduced with an excess of solid sodium dithionite, and the sample was sealed with minimum headspace in a cuvette. CO-saturated buffer was prepared by continuous bubbling with pure CO and was only used after a 30-min preequilibrium. A value of 939 μM was used for the concentration of CO in this saturated buffer at 23 °C (Dean, 1985), and saturation is nearly complete (930 μM) in 30 s (Miura et al., 1991). Additions of CO-saturated buffer with a gas-tight syringe through a Teflon septum were used to vary the concentration of CO in the sample. The visible spectrum was scanned once and again 1 min later to confirm that the sample was completely equilibrated. Then the sample was saturated with CO by bubbling with the pure gas for 30 s to confirm the total P450 content and to monitor the P450/P420 ratio.

Redox titrations were carried out as previously described (Dutton, 1978) under a continuously flushed atmosphere of CO and using CO saturated buffer. The buffer consisted of 0.1 M MOPS–NaOH (pH 7.0), 20 μM pyocyanine, 20 μM methyl viologen, 20 μM benzyl viologen, 20 μM anthraquinone-2-sulfonate, 20 μM 2-hydroxy-1,4-naphthoquinone, 5 μM phenazine methosulfate, and 5 μM phenazine ethosulfate to which was added microsomal protein corresponding to 0.44 μM P450 (Jerusalem artichoke) or 0.13 μM P450 (wheat). At the concentrations used, the redox mediators did not interfere with measurement of the cyt P450 absorbance change at 450–475 nm.

Enzymatic Assays. Reduction of cytochrome P450 by NADPH was assayed in CO-saturated buffer 5 min after the addition of 0.1 mM NADPH. 13(*S*)-Hydroperoxy-9(*Z*),-11(*E*)-octadecadienoic acid (13-HPOD) was prepared as described (Nelson et al., 1990) and provided by Dr. Mark Nelson. 13-HPOD peroxidase activity was assayed by the decrease in absorbance at 235 nm in a solution of 0.1 M sodium phosphate buffer (pH 7.0) and 0.1 mM 13-HPOD and was initiated by the addition of protein; detection of the transient allene oxide was carried out as previously described (Hamberg, 1987; Brash et al., 1988; Song & Brash, 1991). The final products of this reaction were visualized by 2,4-dinitrophenylhydrazine spray following TLC as previously described (Hamberg & Fahlstadius, 1990; Zimmerman & Vick, 1970). NADPH:cytochrome *c* reductase activity was measured by the increase in absorbance at 550 nm in a solution consisting of microsomes, 0.1 M MOPS–NaOH (pH 7.0), 5 μM antimycin A, and 50 μM horse heart cytochrome *c*.

General Measurements. Microsomal cytochrome P450 concentration was determined based on the difference spectrum of the ferrocytochrome–CO complex in saturating CO (Omura & Sato, 1964). Protein was assayed by the method of Bradford (Bradford, 1976) or by the total amino acid content of a hydrolyzed sample (after correction for the water of hydrolysis). Amino acid composition and heme content of the purified tulip P450 were determined as previously described (O'Keefe & Leto, 1989; Appleby, 1969).

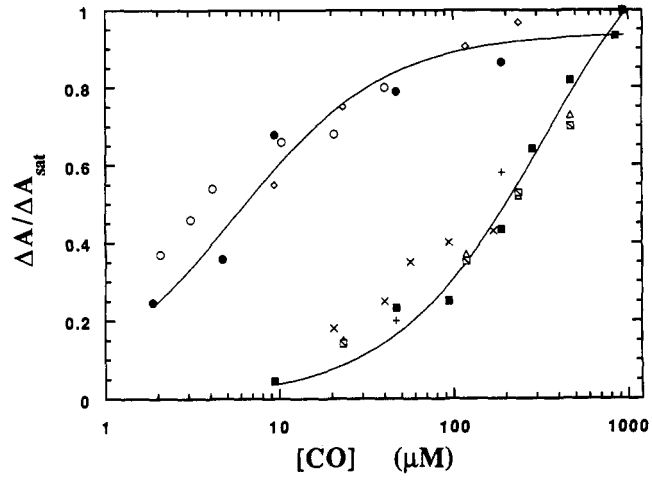

FIGURE 1: CO titration of plant cytochrome P450 preparations. All values were normalized to the $\Delta A_{450-475nm}$ measured in the same sample at saturating CO concentration, ΔA_{sat}. Curve fits for a single affinity constant to avocado and tulip microsomal P450s are shown, with affinity constant values of 1.8×10^5 and 2.9×10^3 M^{-1} and maximum $\Delta A/\Delta A_{sat}$ values of 0.94 and 1.35, respectively. (●) Avocado microsomes; (○) aged Jerusalem artichoke tuber microsomes; (◇) uninjured Jerusalem artichoke tuber microsomes; (■) tulip bulb microsomes; (△) purified tulip P450; (⊠) purified tulip P450 + 5 μM methyl viologen; (×) etiolated wheat coleoptile microsomes; (+) corn germ microsomes. Note that all samples have a data point at 939 μM CO and 1.0 $\Delta A/\Delta A_{sat}$.

RESULTS

Plant cytochromes P450s have been purified from specialized tissue in avocado, Jerusalem artichoke, and tulip (Higashi, 1985; O'Keefe & Leto, 1989; Gabriac et al., 1991). These studies demonstrate that one or two closely related P450s constitute the majority of the microsomal cytochrome P450 complement in these tissues. Nonetheless, the P450s isolated from each of these species are functionally distinct; the Jerusalem artichoke P450 is a *trans*-cinnamate hydroxylase (Gabriac et al., 1991); the avocado enzyme metabolizes xenobiotics, but its endogenous role is most likely that of a monoterpene hydroxylase (O'Keefe & Leto, 1989; Hallahan et al., 1992); and the tulip P450 carries out neither of these activities, and its enzymatic role was previously unknown (Higashi et al., 1985). Our initial observation that tulip microsomal cytochrome P450 did not fully form its ferrocytochrome–CO complex after brief treatment with CO and reductant led to an investigation of the equilibrium properties of this complex. We included cytochromes P450 from several types of tissue in the plant species that have been studied in some detail, in order to compare the CO affinity of the microsomal P450 complement from these tissues.

Figure 1 shows the results of a CO titration of the ferrocytochrome P450 in microsomal fractions from five plant species. An outstanding feature of this graph is that these P450s fall into two distinct categories, with high and low CO affinity. Fits of the data for avocado and tulip microsomal P450 suggest that values for these two equilibrium affinity constants (K) are 1.8×10^5 M^{-1} and 2.9×10^3 M^{-1}. In order to evaluate our techniques, we measured K values of 2.3×10^5 M^{-1} [compared to 3×10^5 M^{-1} (Sono et al., 1986)] and 1.3×10^6 M^{-1} [compared to 2.2×10^6 M^{-1} (Peterson & Griffin, 1972)] for chloroperoxidase and substrate-free $P450_{CAM}$,

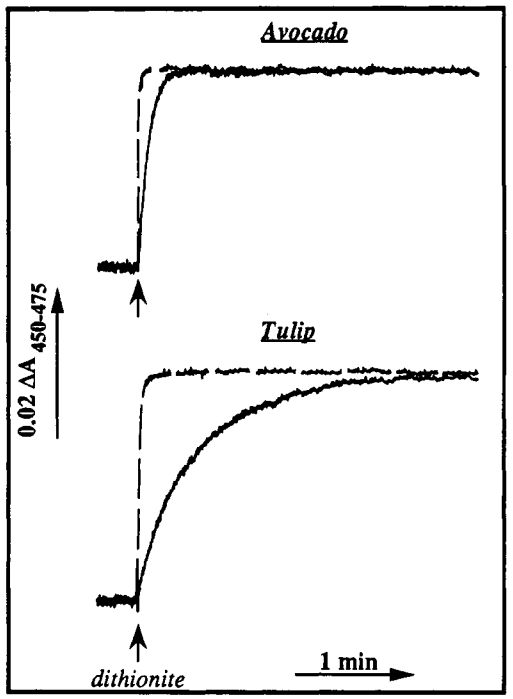

FIGURE 2: Appearance of the 450-nm chromophore following dithionite addition. Samples were ~0.5 μM P450 from avocado or tulip bulb microsomes and were rapidly stirred in CO-saturated 0.1 M MOPS–NaOH (pH 7.0) when 10 mM sodium dithionite was added as indicated. The dashed lines indicate the presence of 5 μM methyl viologen.

respectively. Although all experimental values in Figure 1 are normalized to 1.0 at saturating CO, it is evident for tulip P450 and those like it that the binding of CO is not complete at saturating CO concentration but is only ~74% of the full value calculated from the optimum curve fit. We have not attempted to measure CO binding at pressures exceeding 1 atm. Two equilibrium constants could be fit to some of the data (not shown), but there are not sufficient data to accurately resolve the K values of minor (<20%) P450 forms. For purposes of further discussion, we will categorize the microsomal fractions as containing 'predominantly low CO affinity' and 'predominantly high CO affinity'.

The different CO affinities in Figure 1 were measured in the presence of excess reductant; it was assumed that the final equilibrium of ferrocytochrome–CO formation was reached. However, it is possible that the apparent low CO affinity is a consequence of incomplete or slow reduction of some of the P450s under these conditions. To test for this, we have measured both the rate of dithionite reduction of the P450, and the equilibrium redox potential of two of these plant P450s. Of the microsomes used in this study, only those from avocado and tulip have no significant dithionite-reducible components with spectroscopic changes at 450 nm. It is thus possible to measure reduction of tulip and avocado P450 from the formation of the 450-nm chromophore after addition of dithionite in the presence of saturating CO. Figure 2 shows that the formation of chromophore after dithionite addition is slower with tulip P450 ($t_{1/2}$ is ~5 and ~20 s for avocado and tulip, respectively). Addition of the redox mediator methyl viologen accelerates chromophore formation to the mixing time of this sample (~0.5 s), demonstrating that it is the slow dithionite reduction of the P450 that determines the rate of appearance of the 450-nm chromophore, not the binding of CO to the reduced cytochrome. These results also demonstrate that the final equilibrium formation of P450–CO in tulip microsomes is ~96% complete in 2 min (the minimum time

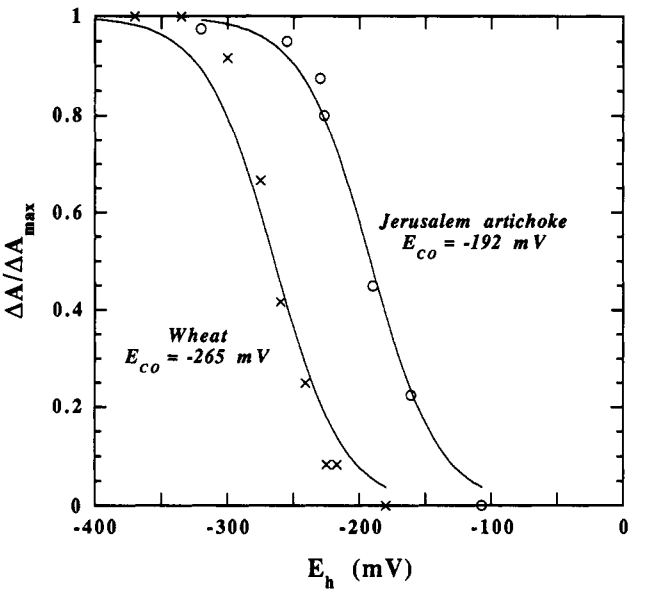

FIGURE 3: Redox titration of Jerusalem artichoke and wheat microsomal cytochromes P450. Values are normalized to the total ΔA observed upon full P450 reduction, ΔA_{max}.

to collect a single data point in Figure 1).

Figure 3 shows the results of a redox titration of the P450–CO in wheat and Jerusalem artichoke microsomes, with curve fits to $n = 1$ redox midpoint potentials (relative to the standard hydrogen electrode) of –265 and –192 mV, respectively. Since it is generally assumed that CO binds only to ferrocytochrome P450 ($P450^{II}$), the only significant microequilibria that contribute to the measured redox potential in the presence of CO are the $P450^{III}/P450^{II}$ redox couple, and the binding of CO to $P450^{II}$. Using the CO equilibrium constants from Figure 1, the redox potentials measured in Figure 3 are related to E, the standard potential in the absence of CO by:

$$E = E_{CO} + \frac{RT}{nF} \ln \frac{1}{K[CO]}$$

where E_{CO} is the midpoint potential measured in the presence of CO at concentration [CO], and K is the affinity constant of the ferrocytochrome–CO complex. In this way, the CO-free standard potentials of –325 and –291 mV were estimated for Jerusalem artichoke and wheat total microsomal P450, respectively. CO-free tulip P450 had previously been found to have a redox midpoint potential of –315 mV by redox titration of the EPR signal (Rich et al., 1975); this would be –341 mV in saturating CO. We conclude that tulip, Jerusalem artichoke, and wheat P450s are about equally difficult to reduce; thermodynamically, all should be reducible by dithionite as well as NADPH, especially in saturating CO. Together with the results on the rate of reduction in the presence of CO (Figure 2), these findings confirm that the experimental technique used in Figure 1 provides an accurate determination of the low CO affinity for the plant ferrocytochromes P450.

As Table I shows, CO affinity correlates with NADPH reducibility of the microsomal P450; only high-affinity P450s exhibit any reduction in the presence of NADPH. From Figure 3, the low CO affinity P450s in both wheat and tulip have sufficiently high redox midpoint potentials, especially in the presence of CO, to be reducible by NADPH (E = –320 mV). The lack of reduction by NADPH may be due to the absence of sufficient levels of NADPH:P450 reductase in tulip and corn germ, as indicated by the low levels of NADPH: cytochrome c reductase activity. However an alternative

Table I: Comparison of Cytochrome P450 CO Affinity with NADPH-Dependent Activities and 13-HPOD Peroxidase in Several Plant Microsomal Fractions

plant source	CO affinity	% NADPH reducible[a]	Cyt *c* reductase (nmol mg^{-1} min^{-1})	13-HPODase (nmol nmol $P450^{-1}$ s^{-1})
Jerusalem artichoke tuber	high	35	128	<39
avocado mesocarp	high	87	63	<2
wheat coleoptile	low	<5	74	2187
tulip bulb	low	<5	2	5800
corn germ	low	<5	9	6200

[a] Measured after 5-min incubation with NADPH, 30-s bubbling with CO, and subsequent 1 min before scanning spectrum.

explanation is necessary to account for the lack of NADPH reducibility in wheat, which has NADPH:cytochrome *c* reductase activity comparable to avocado and Jerusalem artichoke. Table I also shows that those microsomal fractions containing predominantly low CO affinity P450 also exhibit substantial levels of 13-HPOD peroxidase activity, similar to the AOS activity in flaxseed described previously. If this is the physiological role of the predominant P450 in these tissues, these enzymes would not require reducing equivalents and may not even have a site for interaction and electron transfer with an NADPH:P450 reductase. This would account for the lack of NADPH reduction in the microsomes containing predominantly low CO affinity P450s: wheat, tulip, and corn germ. Slow reduction by dithionite in the absence of redox mediators (Figure 2; also with purified protein, not shown) suggests that the heme in tulip P450 is well-insulated from interactions with polar redox active components, consistent with this latter hypothesis.

To further characterize the low CO affinity P450s, we purified from tulip the predominant cytochrome P450 form. This protein was purified essentially as previously described (Higashi et al., 1985), with the addition of a final gel filtration step which allowed substitution of octyl glucoside for the Triton X-100. The complete procedure yielded an electrophoretically homogeneous 48.5-kDa protein with an A_{393}/A_{280} ratio $\geq$ 1.2, with a 44% overall yield of purified P450. No other cytochromes P450 were detected as minor fractions during the preparation. The amino acid composition was essentially the same as previously reported (Higashi et al., 1983), and using this composition to quantify protein, we determined a specific heme content of 18.6 nmol/mg (theoretically 20.6 nmol/mg for a single heme containing the 48.5-kDa protein). The absolute heme content was used to calculate extinction coefficients: 152 mM^{-1} cm^{-1} for the oxidized protein at 393 nm and 74 mM^{-1} cm^{-1} for the ferrocytochrome–CO difference at 450 nm. This latter value is 81% of the of the generally assumed value, 91 mM^{-1} cm^{-1} (Omura & Sato, 1964), a discrepancy consistent with the incomplete (74%) binding of CO at saturating gas concentrations noted earlier.

As shown in Figure 1, purified tulip P450 retains the same CO affinity as found for the entire tulip microsomal P450 complement. Additionally, we measured nearly identical values when this titration was done in the presence of 5 μM methyl viologen, allowing for more rapid redox equilibration. Tulip P450 actively catalyzed the disappearance of 13-HPOD exhibiting enzymatic parameters at 23 °C of 17 μM and 4300 s^{-1} for the K_m and V_{max}, respectively, compared with values from the microsomal preparation of 40 μM and 4000 s^{-1} (assuming total microsomal P450 is involved in turnover). Together these results on the purification and properties of this tulip P450 demonstrate that it is the predominant P450 present in the microsomal fraction and that it appears to be entirely responsible for the CO affinity and 13-HPOD peroxidase activity measured in the microsomal fraction.

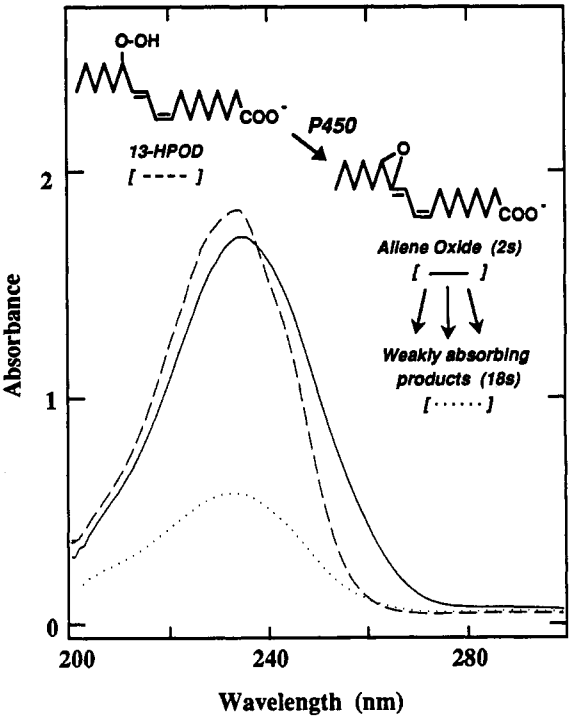

FIGURE 4: Transient appearance of 13-HPOD-derived allene oxide. To 0.1 mM 13-HPOD was added 48 nM (final) purified tulip P450 at 5 °C, and the spectra were recorded at 2-s intervals. The spectra shown are for 13-HPOD and at 2 and 18 s after enzyme addition.

AOS enzymes metabolize 13-HPOD to an unstable allene oxide (Figure 4). The allene oxide formation mediated by purified tulip P450 was detected by its characteristic UV spectrum slightly red-shifted from the parent 13-HPOD (Hamberg, 1987; Brash et al., 1988; Song & Brash, 1991). Subsequent nonenzymatic breakdown led to less UV absorbant products (Figure 4). This measurement is made at lowered temperatures to increase the lifetime of the allene oxide and with high enzyme concentrations to rapidly convert all the substrate to product. The stable products of this reaction catalyzed by purified tulip P450, wheat microsomes, and corn germ microsomes appeared identical on TLC, with a single major product. On the basis of previous identification of this stable major metabolite with corn germ microsomes (Hamberg, 1987; Hamberg & Fahlstadius, 1990), its R_f value on TLC, and the positive 2,4-dinitrophenylhydrazine reaction (Zimmerman & Vick, 1970), we conclude that this major product is the α-ketol, 12-keto-13-hydroxy-9(*Z*)-octadecenoic acid. The observation of this and the transient allene oxide formation demonstrate that the tulip P450 is an AOS and suggest that the low CO affinity P450s in corn germ and wheat are the same enzyme.

The low affinity for CO of the AOS P450s and the lack of reduction by NADPH suggest that these P450s, unlike virtually all of the P450s involved in the reductant requiring O_2-dependent monooxygenase reactions, may have a structural

feature(s) in the distal heme pocket which interferes with binding of CO and, by analogy, O_2. If this were the case, it would be expected that binding of other axial heme ligands would be similarly hindered. We have compared the affinity of three ligands to the ferricytochrome P450 measuring the 'type II' absorption spectra (Jefcoate, 1978) with purified monooxygenase P450 from avocado and the AOS P450 from tulip. Both clotrimazole and 1-phenylimidazole bind very tightly to avocado P450, with affinity constants $>2 \times 10^7$ M^{-1}. These compounds elicited very small changes with tulip P450 from which we estimate an affinity of $<1 \times 10^3$ M^{-1} for each. CN^- binds to avocado P450 with $K = 8.3 \times 10^3$ M^{-1} and to tulip P450 with $K = 26$ M^{-1}. These results confirm that the affinity of tulip ferricytochrome P450 for axial heme ligands is greatly reduced, with at least 300-fold decreased affinity for all of the ligands tested when compared to avocado P450.

Polyclonal antiserum raised against the tulip P450 does not inhibit its 13-HPOD peroxidase activity at serum dilutions up to 1/100. This antiserum strongly and specifically reacts with both microsomal and purified tulip P450 and can be used to detect 1 pmol of this protein on Western blots at serum dilutions of as low as 1/50 000. Nonetheless, Western blots with any of the four other microsomal fractions compared in this study failed to exhibit any antigenic cross-reactivity. This result is similar to that found previously with another antibody raised against the tulip P450. These findings suggest that among different plant species there exists a great deal of antigenic diversity, even among what appear to be catalytically identical enzymes. This is similar to what has been observed with plant monooxygenase P450s, although some examples of antigenically related P450s have been reported (Gabriac et al., 1991; Kochs et al., 1992; O'Keefe et al., 1992).

DISCUSSION

The results presented here demonstrate that the major cytochrome P450 found in microsomal fractions from different tissues in three different species is one with low affinity for CO. In tulip, this protein is an AOS, and similar activity in the other tissues suggests that the low CO affinity P450 is an AOS also. While our sampling is by no means extensive, our findings suggest that a low CO affinity AOS is commonly the predominant P450 in monocot tissues. Although not present among those studied here, an AOS P450 has also been found in dicots, where low 450-nm absorption of the flaxseed P450 (Song & Brash, 1991) suggests that it also has a low CO affinity.

NADPH and O_2-dependent monooxygenase reactions have been studied in a number of monocots, particularly because of their ability to metabolize xenobiotics, especially herbicides. It is especially noteworthy that turnover numbers for plant monooxygenase metabolism of xenobiotics typically range from <1 to 50 min^{-1} (O'Keefe & Leto, 1989; Frear et al., 1991). This can be contrasted with a turnover number for the tulip AOS P450 expressed in the same units of 260 000 min^{-1}. The plant monooxygenase reactions are so slow by comparison that a very small diversion (<0.01%) of AOS heme–oxygen intermediates from the normal mechanistic cycle to oxidize an alternative substrate could be comparable in overall rate to monooxygenase-catalyzed reactions with that substrate. A related result has been noted previously with an avocado P450 that carries out peroxygenative demethylation of *p*-chloro-*N*-methylaniline 10 times faster than the reductase-mediated monooxygenase reaction (O'Keefe & Leto, 1989). These findings suggest that plant P450s are capable of some range of peroxygenative activities at rates that are intermediate between the slower monooxygenase and the very fast peroxidase reactions. It remains to be determined if substrates substantially different from 13-HPOD can be metabolized by the AOS P450s in a peroxygenase reaction, with 13-HPOD, H_2O_2, or another peroxide as cosubstrate.

We have demonstrated that plant P450s can be categorized by large differences in their affinity for heme ligands, including CO, CN^-, and substantial imidazoles. These categories reflect clear functional distinctions. Differences in heme ligand affinities have been investigated earlier in order to study the effect of substrate binding (Peterson & Griffin, 1972; O'Keeffe et al., 1978) and as a means of resolving distinct forms of cytochrome P450 in animal liver (Comai & Gaylor, 1973; Balny & Debey, 1976; Gray, 1978). In none of these studies of heterogeneity was the difference in CO affinity as large as has been observed between plant species here, nor was the CO affinity of any form lower than $\sim 8 \times 10^5$ M^{-1}, nearly 300-fold more than tulip P450. More recently, heterogeneity of CO binding kinetics has been observed with purified cytochrome $P450_{PB}$ (Oertle et al., 1985), and CO binding to microsomal P450s was found to be strengthened by the presence of O_2 (Miura et al., 1991). These factors would not seem to contribute to our results with purified tulip where there is no apparent heterogeneity and O_2 is excluded from the experiments. However, we have not analyzed CO binding/dissociation kinetics with any of the plant P450s; there may be smaller differences overlooked in the simple equilibrium experiments conducted here.

The properties of the AOS P450s described here imply that this class of P450s may have some unique structural features that differentiate it from the reductant-requiring monooxygenase P450s. If there is no site for the introduction of reducing equivalents, then conserved regions of eucaryotic P450s responsible for the binding of reductant and electron transfer to the heme should be missing in these proteins (Nelson & Strobel, 1988; Kalb & Loper, 1988; Stayton et al., 1989). A more clear-cut implication of this work relates to a region that has been defined in the three-dimensional structure of cytochrome $P450_{CAM}$ and is conserved in nearly all cytochrome P450 sequences. The five amino acids comprising this region form a groove in the α-helix that runs across the distal heme face (Raag & Poulos, 1989). This groove is aligned so that a nearly linear Fe-C-O bond is possible in $P450_{CAM}$, and the Fe-C distance is nearly the same as in model compounds. We speculate that the hindered binding of axial ligands in tulip and related AOS P450s must be a consequence of different structure in this region of the protein. The specific attributes of this alteration need to accommodate the observed weaker binding of axial ligands and, by implication, the exclusion of O_2 as unnecessary to the catalytic cycle, yet they need to allow coordination of the fatty acid hydroperoxide and its reaction intermediates.

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