Antigenic Crossreactivity between Bacterial and Plant Cytochrome P-450 Monoxygenases

Cassie B. Stewart and Mary A. Schuler

Department of Plant Biology, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Although cytochrome P-450 monoxygenases mediate critical reactions in plant microsomes, characterization of their activities has been difficult due to their inherent instability and the lack of a crossreacting P-450 antibody. We have surveyed the effects of protein stabilizing agents on t-cinnamic acid hydroxylase (t-CAH), a prominent microsomal P-450, and on total P-450 monoxygenase content. Trans-cinnamic acid is the most effective protecting agent for t-CAH activity. Leupeptin, a broad spectrum protease inhibitor, stabilizes t-CAH activity and increases the apparent P-450 content more than serine protease inhibitors such as phenylmethylsulfonyl fluoride. The combination of t-cinnamic acid and protease inhibitors increase the level of detectable t-CAH activity 4- to 14-fold over the levels detected by previously published procedures. In order to estimate the molecular weights and diversity of the plant P-450 monoxygenases in wounded pea epidermis, we have prepared two polyclonal antibodies against the Pseudomonas putida camphor hydroxylase (P-450mam). One of the heterologous antibodies cross-reacts with constitutive microsomal polypeptides between 52 and 54 kilodaltons and several pea (Pisum sativum L.) mitochondrial proteins between 47 and 48 kilodaltons. The other polyclonal antibody cross-reacts strongly with two wound-induced polypeptides (65 and 47 kilodaltons) and weakly with one constitutive polypeptide (58 kilodaltons). We conclude that at least two subclasses of plant P-450 monoxygenases share common epitopes with the bacterial P-450 enzyme.

Cyt P-450 monoxygenases are key components in the metabolic pathways of prokaryotes, lower eukaryotes, and probably all higher eukaryotes. The ubiquitous distribution of these enzymes is equaled only by the variety of molecules that are their substrates and the number of proteins that fall into this classification. In mammalian cells, there are two broad classes of Cyt P-450 enzymes: the "mitochondrial type," which carry out the highly regio- and stereo-specific hydroxylations involved in steroid biosynthesis, and the "microsomal type" that often display very broad substrate specificities and are involved in detoxification and general catabolic reactions (23). In mammalian tissues, the first type of monoxygenase system, the mitochondrial system, is hormone regulated (34). The second type of monoxygenase system, the microsomal system, includes a large number of P-450 hemoproteins, each of which has broad, but overlapping, substrate specificities. These various P-450 monoxygenases are differentially induced by a variety of exogenous agents, which are related to their respective metabolic activities (2). Characterization of the mammalian P-450 monoxygenases at the molecular level has revealed that the mitochondrial and microsomal P-450 components exhibit a wide variety of mol wt between 46 and 57 kD. (6). The mammalian P-450 proteins, when probed with polyclonal antibodies in radioimmunoassays, demonstrate distinct but limited relatedness. In these comparisons, antibody directed against rabbit P-450LM2 cross-reacted with the rabbit microsomal P-450LM3, the bovine P450cam, and the bovine mitochondrial P-450occ and P-45011b (10). In similar experiments, P-450cam, polyconal antibody cross-reacted with rabbit P-450LM2 and P-450LM4, the bovine mitochondrial P-450occ and P-45011b, and microsomal P-45017a and P-450c21. Immunochemical reactivities have also been demonstrated within subclases of the P-450 isofoms, such as the rat P-450b, P-450e, P-450f subclasses or the P-450c and P-450d subclasses (28). Beyond these examples, antigenic relationships among the P-450 isofoms found in different species are limited, if they exist at all. These results indicate that the mammalian and bacterial P-450 monoxygenases represent a relatively divergent group of proteins in which subsets of enzymes are antigenically cross-reactive because they share structural relatedness. Molecular analysis of the mammalian P-450 genes has demonstrated that within the large P-450 gene family there are subgroups encoding structurally related monoxygenase proteins and other subgroups encoding more distinct proteins (2, 21).

Much less is known about the number of distinct Cyt P-450 isozymes in plant tissue and the mechanisms for control of expression of these genes. The plant Cyt appear to have many similarities to the animal Cyt P-450 enzymes including the most prominent of the P-450 characteristics: oxygen and NADPH-dependences and a 450 nm peak for the ferrous-CO adduct (29, 30). Although mitochondrial P-450 isozymes presumably exist in plant tissues, all of the P-450 activities characterized so far have been localized in microsomal membranes (35). Several important metabolic reactions in plants are thought to be mediated by the P-450 mixed function oxidases. The most prominent P-450 activity detectable in plants appears to be that of t-CAH, which carries out the conversion of t-CA to p-coumaric acid. In another monoxygenase-mediated reaction, kauren, a precursor to the various

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2 Abbreviations: t-CAH, trans-cinnamic acid hydroxylase, G-6-P, glucose-6-phosphate; G-6-P DH, glucose-6-phosphate dehydrogenase; PAL, phenylalanine ammonia lyase; P-450 reductase, NADPH Cyt P-450 reductase; t-CA, trans-cinnamic acid.
gibberellins, is metabolized to 7-β-hydroxy-kaurenoic acid (35). A third set of oxidase-mediated reactions involves the hydroxylation of geraniol and nerol to form 10-hydroxygeraniol or 10-hydroxynerol, two intermediates in the biosynthesis of indole alkaloids (11). Other P-450 mediated reactions occurring in plant microsomes include the C8, C9, C10 and ω-hydroxylations of lauric acid (1, 5, 32).

Low endogenous levels of t-CAH have been detected in a variety of tissues including artichoke tubers (1, 3, 26) and pea seedlings (4). The levels of this enzyme are induced by a wide variety of agents including 2,4-D, manganese, phenobarbital, herbicides (1, 25, 26) and also in response to wounding and ageing (3) and light (4). t-CAH comprises 40% of the total P-450 found in artichoke tuber tissue (27) but only a small fraction of the total P-450 in wounded pea tissue (CB Stewart, MA Schuler, in preparation). The amounts of other microsomal P-450 monooxygenases have not been estimated.

In spite of the vast amount of literature on the reactions mediated by plant monooxygenases, little information exists on the mol wt or the number of P-450 monooxygenases found in plant microsomes. One of the major difficulties encountered in this type of analysis has been the absence of antibodies directed against plant P-450 monooxygenases. Although a 56 kD protein retaining t-CAH activity has recently been purified from artichoke tuber tissue (12), no information exists on the antigenic cross-reactivity of this purified P-450. A tulip bulb P-450 Cyt of unknown function has also been purified (15) but antibody directed against this 52.5 kD protein cross-reacts only with the monomeric proteins found in tulip cultivars and not with microsomal proteins found in Jerusalem artichoke, cauliflower, avocado, or potato (14). Some recent evidence has suggested that a monoclonal antibody directed against the rat P450c isozyme weakly cross-reacts with a 48 kD protein in Phaseolus vulgaris microsomes (8). Apart from these limited immunological studies, no information exists on the relatedness of the plant, bacterial and animal P-450 molecules. To alleviate some of these deficiencies, we have prepared P-450 antibodies that cross-react with constitutive and inducible microsomal proteins and several mitochondrial proteins in pea epicotyls.

Another major difficulty in characterizing these monooxygenase arises from the low levels of P-450 proteins found in plant microsomes. Problems encountered with low protein levels are compounded by the general instability of P-450 proteins in plant extracts during the microsomal isolation procedures. Some of this instability is due to the arrangement of P-450 proteins in the microsomal membrane. Mammalian microsomal P-450s have segments of their structure integrally associated with membranes and other, reasonably large portions of their structure exposed on the cytoplasmic side of the membrane (16). Mild treatment of mammalian microsomes with proteolytic enzymes completely degrades the membrane-associated P-450 proteins (24). Thus, plant P-450 polypeptides are unusually susceptible to degradation by the vacuolar proteases released during tissue homogenization. In this paper, we survey the effects of protein stabilizing agents on the activity of t-CAH, the major plant microsomal P-450, and on the total microsomal P-450 contents. We demonstrate that the low levels of plant P-450 Cyt can be significantly stabilized during microsomal isolation.

**MATERIALS AND METHODS**

**Plant Material**

Pea (Pisum sativum L. Burpee Progress No. 9) seeds were soaked overnight, planted in damp vermiculite, and grown in the dark at 20°C for 6 d. For wound induction studies, epicotyl sections were cut below the apical bud and divided into 1 cm segments, of which 4 to 8 g were incubated for the appropriate time in Petri dishes containing 12 mL of 5 mM sodium phosphate (pH 5.5) at 22°C.

**Microsomal Isolation and Enzyme Assays**

Microsomes were prepared by the procedure of Russell (31) modified to include protease inhibitors and t-CA, the substrate for the reaction. Both types of compounds significantly increase the amount of detectable hydroxylase activity. Plant tissues were ground in a mortar and pestle at 4°C in three volumes of 0.1 M sodium phosphate (pH 7.5), 0.25 mM sucrose, 1 mM EDTA, 10 mM β-mercaptoethanol, 2% Polyclar AT (w/v), 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 1 mM t-CA. The homogenate was filtered through cheesecloth and centrifuged at 4,000g for 5 min. The resulting supernatant was recentrifuged at 13,000g for 10 min and subsequently at 100,000g for 60 min. The microsomal pellet was resuspended in 50 mM sodium phosphate (pH 7.5), 1 mM β-mercaptoethanol, 5 μg/mL leupeptin, 5 μg/mL pepstatin.

t-CAH activity was assayed using HPLC to determine the amount of newly synthesized p-coumaric acid. t-CAH assays were conducted as described by Lamb and Rubery (18) in duplicate 200 μL reaction volumes containing 50 mM sodium phosphate (pH 7.5), a total of 200 μM t-CA, 1.67 mM G-6-P, 0.067 units of G-6-P DH, 0.67 mM NADP+, 1 mM β-mercaptoethanol, and approximately 2 mg/mL protein. Reactions were started with the addition of microsomes and were terminated by bringing the reaction to 0.4 N HCl and 1.25% TCA (w/v). Five nmol o-coumaric acid was added to each sample as an internal standard. The acidified mixture was extracted twice with two volumes of ethyl acetate, the organic phases were evaporated to dryness, and stored in the dark at room temperature until analyzed by HPLC. Total cytochrome P-450 content was determined by the method of Omura and Sato (22).

**HPLC Analyses**

The dried t-CAH assay samples were dissolved in 200 μL 55% water:45% methanol:0.05% TFA (pH 2.6). Ten μL aliquots of each sample were injected into a Varian 2000-series isocratic component HPLC. Separation of p-coumaric acid and t-cinnamic acid was achieved by a modified method of Blume and Saunders (7) using a Varian 5 micron microbore C-18 column. Peak retention times and peak areas were monitored with a Hewlett Packard 3310 integrator.

**Chemicals**

Polyclar AT from General Aniline Film (GAF) Corporation, NADP+, NADH, G-6-P, G-6-P DH, PMSF, β-mercap-
to ethanol, leupeptin, pepstatin, were all purchased from Sigma. HPLC grade methanol and water were used in the HPLC analysis.

**Antibody Preparation and Western Analysis**

Polyclonal antibodies directed against the *Pseudomonas putida* camphor hydroxylase (P-450<sub>cam</sub>) (33) were prepared by injection of purified native P-450<sub>cam</sub> (13) into two rabbits. The resulting antibodies, designated anti-(P-450<sub>cam</sub>) and anti-(P-450<sub>cam</sub>)4 were collected at the same intervals over a 4 month period. One-dimensional gels were prepared and electrophoresed according to Laemmli (17). Western analysis was carried out as described by Burnette (9) blotting to nitrocellulose for 3 h at 100 V using a Bio-Rad Transblot electrophoretic apparatus. Both antibodies directed against the bacterial P-450<sub>cam</sub> protein were incubated with Western blots for 30 min using 1:2000 dilutions or for 60 min using 1:5000 dilutions. Protein-antibody complexes were detected using alkaline phosphatase-linked goat anti-(rabbit IgG) as substrate.

**RESULTS**

To assess the integrity of the microsomal P-450 components in various isolation procedures, we have monitored the level of total P-450 and t-CAH in pea microsomes prepared from nonwounded and wounded pea tissue extracted with various buffers. Wounded pea tissue has up to 3.4-fold higher levels of t-CAH activity than nonwounded pea tissue prepared in the same manner (Table I) and provides a more sensitive measure of the integrity of t-CAH.

In this study, the levels of t-CAH were determined using a modified version of the Lamb and Rubery (18) microsomal isolation followed by HPLC analysis. In our modifications we have included a variety of protein stabilizing agents in the initial grinding buffer. In the presence of these agents, the level of detectable t-CAH in nonwounded and wounded tissue increases 4.5- to 14.4-fold, respectively, over the levels detected with the standard grinding buffer (Table I). t-CA, the substrate for t-CAH, increases the level of detectable t-CAH activity 3-fold in nonwounded tissue. Leupeptin, a broad spectrum protease inhibitor, increases detectable t-CAH activity 1.4-fold. Other protease inhibitors are much less effective in stabilizing t-CAH activity. PMSF, a serine protease inhibitor, and pepstatin, an acidic protease inhibitor, produce only nominal increases in hydroxylase activity (Table I). The stabilizing effects of these agents are synergistic in that the presence of both t-CA and leupeptin elevates activity 4.5-fold. The highest levels of activity attained in our studies are achieved by including three different stabilizing agents, leupeptin, PMSF, and t-CA in the homogenization buffer. Microsomal preparations assayd with all three of these agents contained 5-fold more detectable hydroxylase activity than microsomes prepared by the standard protocol without protein stabilizers. Using these conditions, we find that wound reproducibly increases the level of measurable t-CAH activity 3.4-fold to 103 nmol/h/mg protein. In the absence of these agents, t-CAH induction is at best 1.1-fold. The stabilization of t-CAH activity by t-CA is specific for this hydroxylase. The activities of two other microsomal proteins, NADPH Cyt P-450 reductase and Cyt b<sub>5</sub> are unaffected by the presence of t-CA in the isolation buffer (data not shown).

The additions of these components to the grinding buffer for the wounded pea sections result in t-CAH activities (100–135 nmol/h·mg protein<sup>-1</sup> or 100 nmol/h·g fresh weight<sup>-1</sup>) which are 2-fold higher than the activities previously reported in light-induced pea seedlings (30–60 nmol/h·g fresh weight<sup>-1</sup>); 4) and as high as the t-CAH activity detected in wounded tuber tissue (65–120 nmol/h·mg protein<sup>-1</sup>); 3, 26).

To determine whether these agents help maintain the levels of other P-450s in plant microsomes, we have monitored the total P-450 content of our preparations in carbon monoxide different spectra (22). As shown in Figure 1, microsomes prepared in the presence of leupeptin, t-CA, and pepstatin exhibit significant light at 450 nm even when iso-

**Table I. Stabilization of t-CAH Activity**

<table>
<thead>
<tr>
<th>Additions to Grinding Buffer</th>
<th>t-CAH Activity (nmol/h·mg protein&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<tbody>
<tr>
<td></td>
<td>Nonwounded</td>
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<tr>
<td>Grinding buffer</td>
<td></td>
</tr>
<tr>
<td>+ 1 mm t-CA</td>
<td>6.7</td>
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<tr>
<td>+ 5 μg/mL leupeptin</td>
<td>21.9</td>
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<tr>
<td>+ 1 mm t-CA + 5 μg/mL leupeptin</td>
<td>30.5</td>
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<tr>
<td>+ 1 mm t-CA + 1 mM PMSF</td>
<td>23.6</td>
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<tr>
<td>+ 1 mm t-CA + 5 μg/mL pepstatin</td>
<td>22.4</td>
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<tr>
<td>+ 1 mm t-CA</td>
<td>28.9</td>
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<tr>
<td>+ 1 mM PMSF</td>
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<tr>
<td>+ 5 μg/mL pepstatin</td>
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<td>+ 1 mm t-CA</td>
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<td>+ 5 μg/mL leupeptin</td>
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**Figure 1. CO difference spectra.** The total P-450 content in pea microsomes was determined by carbon monoxide difference spectra (22). Microsomes were prepared in the presence of t-CA, leupeptin, and pepstatin from either nonwounded or 12 h wounded pea epicotyl sections and assayed immediately for total P-450 (22) and protein (19). The P-450 content is calculated using an extinction coefficient of 91 mm<sup>-1</sup> cm<sup>-1</sup> for the wavelength pair (450 and 490 nm).
lated from noninduced tissue. Under conditions where these components stabilize t-CAH activity 4- to 14-fold, the total P-450 content measured by CO difference spectroscopy increases 1.1- to 1.2-fold upon wounding. Using the optimal isolation conditions, wounded epicotyl sections contain 75 to 110 pmol P-450/mg microsomal protein. Nonwounded sections contain 67 to 90 pmol P-450/mg microsomal protein. Thus, wounding induces relatively small increases (10–20%) in the total P-450 content of the microsomal membranes.

To estimate the mol wt range for plant P-450 monooxygenases, we prepared two antibodies directed against the Pseudomonas putida camphor hydroxylase (P-450cam) (33). To characterize the level of cross-reactivity between each of the bacterial P-450cam antibodies (anti-[P-450cam,3], anti-[P-450cam,4]) and plant P-450 monooxygenases, microsomal proteins were prepared from wounded and nonwounded tissue in the presence of leupeptin, and l-cinnamic acid and electrophoresed on one-dimensional acrylamide gels (17). In this analysis, the wounded protein sample contains the equivalent of 110 nmol/mg protein·h⁻¹ t-cinnamic acid hydroxylase activity and 4.5 pmol of total P-450 in 60 µg of microsomal protein. The nonwounded sample protein contains 25 to 30 nmol/mg protein·h⁻¹ t-CAH activity in 60 µg of microsomal protein. Western analysis (9) using the anti-(P-450cam,4) antibody on this amount of protein indicates that constitutive polypeptides in a tight doublet at 52.5 to 53.5 kDa cross-react with this antibody (Figs. 2 and 5). In addition, polypeptides at 58.0 and 47.5 kDa weakly react with the anti-(P-450cam,4) antibody. On Western blots probed with the second antibody, anti-(P-450cam,3), wound-induced polypeptides at 63.5 to 65.5 kDa and 47.5 kDa and a constitutive polypeptide at 58.0 kDa cross-react with the antiserum. In some gels, the 65.5 kDa has been resolved into two bands at 65.5 and 67 kDa.

To determine the cellular locations of the 47 to 53 kDa cross-reacting polypeptides, we have fractionated the mitochondrial, microsomal, and soluble components of the tissue homogenate (Fig. 3). Western blot analysis using anti-(P-450cam,4) antibody indicates that the major proteins detected with this antibody are located in microsomal membranes. Mitochondrial proteins of 47.5 and 48 kDa weakly cross-react with the antibody.

The proteins used for the previous analyses have been prepared using leupeptin, pepstatin, and t-CA. Because some components stabilize all P-450 monooxygenases and others preferentially stabilize t-CAH, we have analyzed the pattern of cross-reactive polypeptides present in microsomes prepared with and without these protein stabilizing agents. The pattern of polypeptides analyzed under these conditions demonstrate the general instability of the plant P-450 polypeptides. As visualized in Figure 4, the absence of protease inhibitors and t-CA in the homogenization buffer diffuses the signals for the 47.5 kDa and 65 to 67 kDa wound-induced polypeptides which suggests that these proteins are partially degraded in the absence of protease inhibitors and t-CA. Although protease inhibitors do little to improve the Western signal (lanes 5 and 6), addition of t-CA (lanes 3 and 4) improves the resolution of the 47.5 kDa and 65 to 67 kDa polypeptides. By including both t-CA and protease inhibitors in the isolation medium, we demonstrate the 47.5 and 67 kDa species increase three to fivefold in wounded tissue. t-CA acid and protease inhibitors have less effect on the constitutive polypeptides detected with the anti-(P-450cam,4) antibody than on the wound-induced polypeptides detected with anti-(P-450cam,3) (Fig. 4). Omission of both components significantly limited detection of the 52.5 and 53.5 kDa polypeptides in control tissue. The relative signals for the 47.5 and 67 kDa polypeptides obtained with the anti-(P-450cam,3) antibody after wounding and addition of t-CA strongly parallel the increases in t-CAH activity obtained in Table I. In contrast, the polypeptides detected with anti-(P-450cam,4) antibody are inducer and t-CA-independent suggesting that this antibody cross-reacts with P-450 monooxygenases other than t-CAH.

To determine the kinetics of induction for the cross-reactive polypeptides, microsomal proteins were prepared for Western analysis at 0, 2, 3, 6, 8, and 10 h after wounding. In this time course, t-CAH activity is induced fourfold between 6 and 10 h after excision after a characteristic lag period (CB Stewart, MA Schuller, in preparation). On Western blots analyzed with the anti-(P-450cam,3) antibody, increased amounts of the 63.5 to 67 kDa protein are first observed at 3 h (Fig. 5). Accumulation of the 47.5 kDa polypeptide begins between 3 and 6 h after wounding. None of the proteins detected with anti-(P-450cam,4) antibody vary during this time course.

DISCUSSION

We have demonstrated that a number of reagents stabilize t-CA hydroxylase activity during the microsomal isolation
enzyme-reactive proteins detected on Western blots is clearly enhanced by the addition of these inhibitors.

Using these modified microsomal isolation conditions, the levels of t-CA hydroxylase detected in wounded pea tissue are at least as high as the activities previously detected in aged artichoke tuber tissues (3, 26). In the presence of these agents, wounding reproducibly induces the level of t-CAH 3.4-fold over the endogenous levels found in pea seedlings. The microsomal P-450s are sufficiently stabilized so that total P-450 content can be measured by CO difference spectra (22). Previously, the low apparent levels of P-450 in pea microsomes prevented this type of analysis. As seen in Figure 1, wounding increases the total P-450 content of the microsomes 1.1-fold indicating that a select subset of the P-450 monooxygenases, including t-CAH and possibly others in the lignin biosynthetic pathway, are induced in response to wounding.

Our measurements of t-CAH activity and total Cyt P-450 content allow estimation of the turnover number for pure enzyme and the actual fraction of t-CAH present in wounded and control microsomes. In these calculations, \( X \) equals the turnover amount of pure enzyme in units of nmol product produced per minute per nmol of pure t-CAH. \( T \) and \( T' \) represent the total P-450 content per mg total protein of control and induced preparations, respectively, and \( A \), \( A' \) and \( O \), \( O' \) represent the corresponding measured activity (in units of nmol product produced per minute per mg of total protein) and the content of all other cytochrome P-450s (in units of nmol per mg total protein). Then: \( T = \frac{A}{X} + O \) and \( T' = \frac{A'}{X} + O' \) with \( T = 0.078 \) nmol mg\(^{-1}\), \( T' = 0.0925 \) nmol mg\(^{-1}\), \( A = 0.5 \) nmol min\(^{-1}\) mg\(^{-1}\), and \( A' = 1.7 \) nmol min\(^{-1}\) mg\(^{-1}\).

If we assume that the total level of most other P-450s is not altered by wounding, then \( O = O' \) and the value of the turnover number \( X \), can be calculated to be 83 min\(^{-1}\). Using this value, the fraction of t-CAH in control and wounded microsomes is \( \frac{A}{X}/T = 7.7% \) and \( \frac{A'}{X}/T' = 22\% \), respectively. This turnover number is within the range determined for hepatic microsomal P-450 systems that have turnover numbers from 1 to 100 min\(^{-1}\), and an increase in t-CAH of 14% upon wounding is consistent with observed protein levels. If P-450s other than t-CAH are degraded, such that \( O' \) is less than \( O \), the derived turnover estimate would be correspondingly decreased.

The turnover rate for t-CAH purified from Jerusalem artichoke tubers has been reported as 0.088 nmol min\(^{-1}\) nmol P-450\(^{-1}\) (18). Unfortunately, the partially purified P-450 preparation used for this study had a specific P-450 content of 1.7 nmol mg\(^{-1}\). Using the reported mol wt of 56 kD, pure t-CAH should have a specific content of 17.9 nmol mg\(^{-1}\) suggesting that the preparation is only 9.5% pure. In addition, the assay conditions reported were not limited by the P-450 component, but rather by the levels of NADPH-dependent P-450 reductase present, since the reported hydroxylase activity decreased 2.51-fold (to 0.035 min\(^{-1}\)) when the reductase concentration was decreased 2.49-fold (from 0.046 units to 0.0185 units). We believe that the careful stabilization of microsomal protein by the procedures described here yields a more reasonable estimate of the t-CAH turnover number.

To evaluate the P-450 monooxygenase components in microsomal membranes, we have probed protein blots with

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**Figure 3.** Western blot analysis of microsomal, mitochondrial, and soluble proteins. Wounded pea epicotyls were ground in the presence of the three stabilizing agents, nuclei, and cell debris were eliminated by centrifuging at 4,000 g and the remaining supernatant was fractionated as described in "Materials and Methods." Sixty \( \mu \)g protein from each cellular fraction were electrophoresed on 10% SDS-acrylamide gels, blotted to nitrocellulose (9), and probed for 60 min with preimmune serum (lanes 1-4) or anti-(P-450\(_{cam}\)) antisera (lanes 5-8) at 1:5000 dilutions. Lanes 1 and 5, mitochondrial fraction (13,000 g pellet); lanes 2 and 6, microsomal fraction (100,000 g pellet); lanes 3 and 7, soluble fraction (100,000 g supernatant); lanes 4 and 8, 0.1 \( \mu \)g purified P-450\(_{cam}\) protein.
PLANT CYTOCHROME P-450 MONOXYGENASES

Figure 4. Western blot analysis of microsomal proteins prepared with protein stabilizers. Microsomal proteins were prepared from nonwounded or 12 h wounded tissue in the presence of either 1 mM t-CA or protease inhibitors (5 μg/mL leupeptin, 5 μg/mL pepstatin) or both sets of components as designated at the top. Sixty μg protein from each microsomal preparation were electrophoresed on duplicate 10% SDS-acrylamide gels, transferred to nitrocellulose, and cross-reacted for 30 min with 1:2000 dilutions of the anti-(P-450CAM) antisera (left panel) or anti-(P-450CAM4) antisera (right panel). Both panels: lane 1, 60 ng purified P-450CAM protein; lane 2 in left panel, prestained mol wt standards (73, 45, 33 kD), lanes 3 to 6, 60 μg microsomal protein from 12 h wounded tissue isolated with t-CA and/or protease inhibitors as designated at the top; lanes 7 and 8, 60 μg microsomal protein from nonwounded tissue, prepared with or without t-CA and protease inhibitors as indicated.

Figure 5. Western analysis of P-450 monooxygenases after wounding. Microsomal proteins were prepared for Western analysis at 0, 2, 3, 6, 8, 10 h after wounding in the presence of t-CA, leupeptin, and pepstatin. Fifty μg protein from each microsomal sample were electrophoresed on duplicate 10% SDS-acrylamide gels blotted to nitrocellulose (9) and probed for 30 min with 1:2000 dilutions of the anti-(P-450CAM3) or anti-(P-450CAM4) antisera. In each panel: lane 1, purified P-450CAM protein (1 μg, left panel; 60 ng, middle and right panels); lane 2, mol wt standards; lanes 3 to 8, microsomes prepared at designated times after wounding: (3) 10 h, (4) 8 h, (5) 6 h, (6) 3 h, (7) 2 h, (8) 0 h. Left, Coomassie blue stained gel; middle, Western blot probed with anti-(P-450CAM3) antisera; right, Western blot probed with anti-(P-450CAM4) antisera.
antibodies directed against the bacterial P-450<sub>cam</sub> protein. Western blot analysis indicates that two groups of pea microsomal polypeptides are structurally related to bacterial P-450<sub>cam</sub>. The most interesting group of cross-reacting polypeptides, detected with anti-(P-450<sub>cam</sub>) antibody, includes two wound-inducible polypeptides at 47.5 and 67.0 kDa and a constitutive species at 58.0 kDa. The second group of reactive proteins at 52.5 to 53.5 kDa, and some weakly reactive proteins at 47.5 and 58.0 kDa, are constitutively expressed in pea microsomes. Although mitochondrial P-450 isozymes presumably exist in plant tissues, all the plant P-450 activities previously characterized reside in microsomal membranes (11). Using the P-450<sub>cam</sub> antibodies, we have detected several P-450 cross-reacting polypeptides in the mitochondrial fraction between 47 and 48 kDa, but more careful gradient fractionation is needed to accurately estimate the amounts of these polypeptides in the mitochondrial membranes.

All of the microsomal polypeptides detected with the anti-(P-450<sub>cam</sub>) antibody fall in the size range reported for the bacterial and mammalian P-450 proteins (46–57 kDa; 6). The variations in the mol wt and degree of cross-reactivity are strongly reminiscent of the size and immunological variations seen in the mammalian P-450 monooxygenases. The level of antigenic relatedness between these putative plant P-450 monooxygenases and the bacterial P-450 is quite extensive because the plant polypeptides can be detected in short incubations with very dilute solutions of the P-450<sub>cam</sub> antiserum. The different patterns of cross-reactivity obtained with each antiserum suggest that the polyclonal antibodies are directed against different, discrete epitopes and that relatively few epitopes have been conserved in plant and bacterial P-450 monooxygenases. Clearly, subclasses of the plant P-450 monooxygenases exist which are as divergent in their structure as their mammalian P-450 counterparts.

Because microsomal membranes contain many P-450 monooxygenases in addition to t-CAH (1, 5, 32), we have analyzed the cross-reacting polypeptides in the presence of t-cinnamic acid, which selectively stabilizes t-CAH activity, and protease inhibitors, which stabilize P-450s and other microsomal components. t-CAH, the substrate for t-CAH, stabilizes the 67 and 47.5 kDa wound-induced polypeptides detected with the anti-(P-450<sub>cam</sub>) antiserum. The relative signals obtained for these polypeptides with anti-(P-450<sub>cam</sub>) strongly parallel the increases in t-CAH activity obtained after wounding or the addition of t-CAH during homogenization. Our collective results circumstantially suggest that t-CAH is one of the wound-induced polypeptides at 47.5 or 67 kDa detected with anti-(P-450<sub>cam</sub>) antibody. Although the P-450<sub>cam</sub> antibody does not inhibit t-CAH activity in intact microsomes (data not shown), we do not preclude this possibility because this polyclonal antibody recognizes a limited set of epitopes. If these epitopes lie distal to the catalytic site or within the microsomal membranes, antibody reactions may fail to block t-CAH in vitro.

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LITERATURE CITED