Time Course of Induction of Cytochrome P-450, NADPH-Cytochrome c Reductase, and Cinnamic Acid Hydroxylase by Phenobarbital, Ethanol, Herbicides, and Manganese in Higher Plant Microsomes

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ABSTRACT

The mixed function oxidase trans-cinnamic 4-hydroxylase, cytochrome P-450, cytochrome b5, and NADPH-cytochrome c (P-450) reductase were measured in microsomes from aging artichoke tuber slices exposed to manganese, ethanol, phenobarbital, and the herbicides Chloro-IPC, Dichlobenil, and Monuron. Although the microsomal hydroxylating complex is already induced by the slicing and aging process, 25 millimolar MnCl2, 4 millimolar phenobarbital, and 300 millimolar ethanol caused a marked increase of hydroxylase activity and cytochrome P-450 content and shifted their time course. The herbicides, 200 micromolar Dichlobenil and 200 micromolar Monuron, were less effective. Chloro-IPC was slightly inhibitory. NADPH cytochrome c reductase was significantly increased only in phenobarbital-treated slices. Cytochrome b5 was generally the least affected among the parameters being measured. The mechanisms by which these compounds increase cytochrome P-450 content and hydroxylase activity are not yet defined.

However, the induction (i.e. increase of spectrophotometrically measurable Cyt) of Cyt P-450 in higher plant tissues has been demonstrated only in response to wounding in Jerusalem artichoke (2) and potato tuber tissues (19) and in response to light in pea seedlings (3).

Recently, various xenobiotics have been shown to increase Cyt P-450 concentration in Jerusalem artichoke tuber tissues (20). Here the time course of Cyt P-450, Cyt b5, and CA4H and NADPH-Cyt c reductase activities in aging Jerusalem artichoke tuber tissues exposed to phenobarbital, herbicides, and Mn ions are reported.

MATERIALS AND METHODS

Jerusalem artichokes tubers (Helianthus tuberosus L. cv. Blanc commun), grown locally, were stored in polyethylene bags at 4 C in darkness. The preparation of tuber tissue slices was essentially the same as described previously (2). One-mm thick slices (100 g fresh weight) were incubated in the dark at room temperature in 2-liter Erlenmeyer flasks with 1.5 liters distilled H2O or distilled H2O containing the compounds to be tested. When needed, the pH of the solution was adjusted to 7 with HCl or NaOH. The use of organic solvents was avoided since they alter the induction pattern. The incubation medium was vigorously bubbled with a stream of hydrated and filtered air. The herbicides were added as an ethanol solution to the Erlenmeyer flasks, the alcohol was evaporated, H2O was added, the flasks were shaken for 1 h to dissolve the compound, and tuber slices were added to each flask. Monuron, Dichlobenil and Chloro-IPC were obtained from Serva, Heidelberg (GFR).

For preparation of microsomal pellets, approximately 30 g tuber tissue were homogenized with an Ultra-Turrax homogenizer or a Moulinex mixer in 4 volumes 0.1 M Na-phosphate (pH 7.4) containing 15 mm 2-mercaptoethanol and 2 g insoluble PVP (Polyclar AT). The homogenate was filtered through four layers of cheesecloth and centrifuged 15 min at 10,000g. The resulting supernatant was centrifuged 60 min at 100,000g. The 100,000g pellets were resuspended in 2 ml extraction buffer containing 30% glycerol, frozen, and stored at −20 C until used. There was no apparent loss of enzyme activities or Cyt content over several weeks.

Cinnamic acid hydroxylase was assayed by a procedure modified from Russell (21). In a final volume of 200 μl, the reaction mixture contained: 1.5 mM NADP+, 4.7 mM glucose-6-P, 0.4 units glucose-6-P dehydrogenase, 0.1 to 1 mg microsomal protein, 21 μM trans-[3-14C]cinnamic acid (58 mCi/mmol) (CEA, France), and enough (75 to 300 μM) trans-cinnamic acid to ensure linearity of...
the reaction during the assay. After 15 min preincubation at 25 °C of the complete system less substrate, the reaction was initiated by the addition of cinnamic acid. The reaction was stopped after 20 min by the addition of 20 μl 4 N HCl followed by carrier cinnamic and p-coumaric acids (7 μmol each in 10 μl ethanol). Precipitated proteins were removed by centrifugation and 100 μl supernatant were directly spotted on fluorescent silica TLC plates. The plates were developed in a toluene/acet acid/water (6:7:3) mixture. p-Coumaric acid was located under UV light or by radiochromatogram scanning (Berthold, model LB 2723) and scraped for scintillation counting (Intertechnique model SL 4000).

NADPH-Cyt c reductase was measured by the method of Sottocasa et al. (26) using a mm extinction coefficient of 29.5 at 550 nm for Cyt c.

Spectrophotometric measurements of hemoproteins were performed at 16 °C in a Cary 118 C instrument. Cyt P-450 and Cyt b₅ were measured by the methods of Omura and Sato (16). The following extinction coefficients were used: 91 mm⁻¹ cm⁻¹ for ΔA₄₉₀-₄₄₀ for Cyt P-450 and 185 mm⁻¹ cm⁻¹ for ΔA₄₂₄-₄₅₉ for Cyt b₅.

Microsomal protein content was estimated by the method of Schacterle and Pollock (23).

As reported previously (2), the pattern of development of microsomal activities varies during storage of the tubers. There is an increase in the time needed to reach the maximum and a decrease of the absolute values obtained. However, the pattern of change of microsomal activities in tissues exposed to exogenous compounds relative to that in H₂O controls remains essentially the same.

RESULTS

Hydroxylation of cinnamic acid results from the activity of a membrane bound complex of enzymes (Fig. 1; refs. 1, 2, and 6). The terminal oxidase Cyt P-450 binds both cinnamic acid and molecular oxygen. Once bound, oxygen is activated by reducing equivalents transferred from NADPH and/or NADH via two flavoproteins. Concentrations of Cyt P-450 and b₅ activities of NADPH-Cyt c (P-450) reductase and of NADH-Cyt c (b₅) reductase, and the overall activity of the complex as expressed by the rate of formation of p-coumaric acid can be measured. Since the participation of NADH-Cyt b₅ reductase in the in vivo activity of the enzyme is still a matter of speculation, this parameter is not reported here.

Effect of Mn Ions. Mn ions promote the production of tannin-like compounds in various plants. This effect was first ascribed by Engelsma (7) to a specific induction of phenylalanine ammonia-lyase by the metal. Subsequently, it was shown that Mn promoted also CA4H activity in Jerusalem artichoke tuber tissues (6). The
effect of various Mn concentrations on the microsomal hydroxylating complex in this material was investigated. The effect was highest in tissues exposed to 25 mM MnCl₂. The time courses of Cyt P-450, Cyt b₅, CA4H, and NADPH-Cyt c reductase are shown in Figure 2. At the time of slicing, tuber tissues are devoid of CA4H activity and contain very low Cyt P-450 levels and some NADPH-Cyt c reductase and Cyt b₅. The subsequent induction of enzyme activities and Cyt contents by the slicing and aging process was totally inhibited by 20 μM cycloheximide (2). In tissues exposed to Mn, biphasic induction patterns of CA4H and Cyt P-450 were observed. Apparently, in the first 24 h, the effect of the metal superimposes that produced by wounding. CA4H and Cyt P-450 culminated at 96 h of aging and decreased thereafter. NADPH-Cyt c reductase was not induced but rather time-shifted, the maximum being reached after 48 h. Cyt b₅ was considerably less induced by Mn than Cyt P-450.

Effect of Phenobarbital. Phenobarbital is a typical inducer of Cyt P-450 enzymes in mammalian liver. Electron transport, time course of Cyt P-450, and development of the ER are affected by the barbiturate (17). Maximal effect on the hydroxylating complex was obtained in our material with 4 mM phenobarbital solutions. Low solubility of this compound at neutral pH precluded the use of higher concentrations. Patterns of change in microsomal activities observed when tissues were exposed to 4 mM phenobarbital are shown in Figure 3. During the first 24 h, both wounding and phenobarbital contribute to the response. Cyt P-450 reaches a maximum 60 h after slicing. Its evolution was not exactly paralleled by that of CA4H. This could be due to the synthesis of new Cyt P-450 species not involved in the hydroxylation of cinnamic acid. The activity of NADPH Cyt c reductase was enhanced, but its time course was not modified. Evolution of Cyt b₅ was not significantly affected by phenobarbital.

Previously it was found that various alcohols increase the microsomal hydroxylating system from Jerusalem artichoke tissues (20). The effect was highest in tissues treated with 300 mM ethanol. Ethanol caused a time course (not shown) similar to that observed in phenobarbital-treated tissues, but a generally lesser increase. CA4H, Cyt P-450, and Cyt b₅ were stimulated by 90, 110, and 30%, respectively; NADPH-Cyt c reductase was only slightly enhanced.

Effect of Herbicides. The ability of three herbicides to modify the activity and time course of induction of the hydroxylating system from tuber tissues was investigated. These compounds were not tested at concentrations above 200 μM in order to avoid the use of organic solvents which alter the induction patterns. Figure 4 shows the evolution of enzyme activities and Cyt content in tissues exposed for 24, 48, and 72 h to Dichlobenil, Monuron, and Chloro-IPC. Chloro-IPC was without effect or slightly inhibitory. In contrast, Monuron and Dichlobenil enhanced CA4H activity and Cyt P-450 content. Dichlobenil did not modify the induction pattern produced by wounding, but Monuron clearly stimulated Cyt P-450 and CA4H activity and time-shifted their induction.

**DISCUSSION**

This study was undertaken (a) to evaluate the possibility that Cyt P-450 enzymes are induced by xenobiotics in higher plants as they are in microorganisms and animals and (b) to obtain Cyt P-450 yields high enough to allow the solubilization and resolution of the plant microsomal hydroxylating system. Our findings provide evidence that ethanol, Mn, and foreign compounds increase Cyt P-450 and related monooxygenase activities in higher plant microsomes. This effect is not restricted to the CA4H. The in-chain hydroxylating lauric acid hydroxylase recently discovered

**Fig. 3.** Time course of appearance of CA4H, NADPH-Cyt c reductase, Cyt P-450, and Cyt b₅ in tissues exposed to phenobarbital. Tubers were sliced and aged on H₂O (■—■) or on 4 mM phenobarbital solutions (▼—▼).
in the same plant tissue (22) is also increased by these compounds (unpublished). The products tested have widely differing chemical structures and other compounds, e.g. 2,4-D or the porphyria inducer allylisopropylacetamide, provoke similar effects (unpublished). The mechanisms by which these increases are mediated are unknown and may well be different considering the various induction patterns elicited by the compounds tested. It is difficult, however, to determine whether all these differences pertain to the induction mechanism or to penetration rates or toxic side effects of the inducers.

Mn appears to be a potent inducer of the plant mixed function oxidases. This is surprising, in view of the general toxicity associated with Mn in many plants (for a review, see Ref. 9) and contrasts with the inhibitory effect of most divalent cations on Cyt P-450 induction in animals. Since Mn acts as cofactor in a number of enzymes, the induction observed could be a secondary effect of the modulation of other enzymes by the metal. It was recently reported (5) that in mammals Mn prevents the induction of heme oxygenase, the microsomal enzyme that catalyzes the rate-limiting step in the catabolism of heme. Alternatively, Mn may be implicated in the production of activated oxygen species. The metal undergoes cyclic redox changes during the water splitting process of photosynthesis and is also associated to fungal superoxide dismutases. A total lack of induction was observed when the tubers were sliced under N2 atmosphere. Addition of specific quenchers or scavengers of O2-excite states to the culture medium could provide insight into the mechanism of action of this metal.

Little is known of the effect of barbiturates on plant metabolism. They interfere with mitochondrial electron transport (13) and inhibit germination in rice seeds exposed to low O2 concentrations (14). Phenobarbital is the sole compound among those tested here which increased markedly the NADPH-Cyt c reductase. Concomitant induction of Cyt P-450 and of its reductase is also a characteristic feature of phenobarbital induction in animal tissues, as opposed to the 3-methylcholanthrene-type inducers, which do not affect the reductase level.

A great number of pesticides are inducers and/or substrates of microsomal Cyt P-450 enzymes in mammals and in insects. Cyt P-450-catalyzed hydroxylation or oxidative dealkylation of these compounds is often the first step leading to their detoxication and elimination. There are numerous reports indicating that hydroxylated or N-dealkylated compounds are formed during metabolism of pesticides by plants (8). Because Cyt P-450 content was enhanced by Monuron and Dichlobenil, it is tempting to assume that the microsomal mixed function oxidases play a role in the detoxication of and the resistance to certain pesticides by plants. It is already known that Monuron is N-demethylated by the microsomal fraction from cotton seedlings (10). The non-herbicide xenobiotic p-chloro-N-methylaniline is also demethylated in a Cyt P-450-dependent reaction by a castor bean microsomal preparation (27). Also relevant in this context is the fact that 4-OH-2,5-dichlorophenoxycetic acid and 4-OH-3,5-dichlorophenoxycetic acid are formed in soybean tissues exposed to 2,4-D (8). Chloride migration from position 4 to position 5 or 3 could occur by means of an "NIH shift," i.e. the substrate on an aromatic ring at the site of hydroxyl attack is retained in the final product by migrating to an adjacent carbon atom (11). This mechanism is typical of ring hydroxylation by a monoxygenase.

Besides, the possible role of Cyt P-450 in detoxication and selective resistance of plants toward pesticides, another aspect of the plant-pesticide relationship should be considered. Because Cyt P-450 enzymes intervene in the titer of plant hormones and synthesis of cell wall and cuticle components (12, 24, 25) exposure of plants to pesticides may result, even in the absence of acute toxic symptoms, to qualitative and quantitative changes affecting major metabolic pathways.

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