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Two cytochrome *P*-450 isoforms catalysing *O*-de-ethylation of ethoxycoumarin and ethoxyresorufin in higher plants

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The *O*-dealkylating activities of 7-ethoxycoumarin *O*-de-ethylase (ECOD) and 7-ethoxyresorufin *O*-de-ethylase (EROD) have been fluorimetrically detected in microsomes prepared from manganese-induced Jerusalem artichoke tubers. Cytochrome *P*-450 dependence of the reactions was demonstrated by light-reversed CO inhibition, NADPH-dependence, NADH–NADPH synergism and by use of specific inhibitors: antibodies to NADPH–cytochrome *P*-450 reductase, mechanism-based inactivators and tetcyclasis. Apparent K_m values of 161 μM for 7-ethoxycoumarin and 0.4 μM for 7-ethoxyresorufin were determined. *O*-De-ethylase activity was also detected in microsomes prepared from several other plant species, including wheat, maize, tulip, avocado and *Vicia*. ECOD and EROD were low or undetectable in uninduced plant tissues, and both activities were stimulated by wounding or by chemical inducers. Two distinct cytochrome *P*-450 isoforms are involved in ECOD and EROD activities since (1) they showed different distributions among plant species; (2) they showed contrasting inhibition and induction patterns; and (3) ECOD but not EROD activity was supported by cumene hydroperoxide.

INTRODUCTION

The number of reported substrates of plant cytochrome *P*-450 mono-oxygenases increases steadily. The reactions catalysed are mainly hydroxylations (Durst, 1990) and in some cases epoxidations (Salaün *et al.*, 1989) or *N*-demethylations (Frear *et al.*, 1969; Dohn & Krieger, 1984; Fonné-Pfister *et al.*, 1988). Until now, no *O*-dealkylation or heteroatom oxidations have been reported.

Rapid and sensitive tests to measure the *O*-de-ethylations of 7-ethoxycoumarin and 7-ethoxyresorufin were described respectively by Ullrich & Weber (1972) and by Burke & Mayer (1974). These tests have since been extensively used to characterize and monitor cytochrome *P*-450 isoforms in mammals (Burke *et al.*, 1985), birds (Rivière, 1980; Rivière *et al.*, 1985), fish (Elcombe & Lech, 1979; Klotz *et al.*, 1983) and insects (Reidy *et al.*, 1987; Lee & Scott, 1989). The sensitivity of these tests makes them suitable for fine mechanistic studies (Miwa *et al.*, 1984, 1985) as well as for pollution monitoring (Addison & Edwards, 1988).

In this paper we make use of these tests, adapted to the plant material, (1) to demonstrate the ability of plant cytochrome *P*-450 to catalyse *O*-dealkylation, and (2) to characterize two independent *P*-450 isoforms in induced Jerusalem artichoke microsomes. Kinetic parameters, susceptibility to inhibitors, and plant distributions and induction patterns allow the differentiation of these two forms.

MATERIALS AND METHODS

Chemicals

7-Ethoxycoumarin, 7-ethoxyresorufin, 7-hydroxycoumarin (umbelliferone), resorufin and most other chemicals were purchased from Sigma. Aminobenzotriazole (ABT) and 11-dodecenoic acid (11-DDNA) were gifts from Professor P. A. Ortiz de Montellano, University of California, San Francisco, CA, U.S.A.; chlorsulfuron was a gift from Du Pont, Wilmington, DE, U.S.A.; chlorotoluron from Ciba–Geigy, Basel, Switzerland; diclofop from Hoechst A. G., Frankfurt-am-Main, Germany; and tetcyclasis from BASF A. G., Ludwigshafen, Germany.

Plant materials

Jerusalem artichoke (*Helianthus tuberosus* L., var. Blanc commun) tubers were sliced, washed and extracted immediately or incubated in aerated distilled water for 24 h in the dark to achieve wounding-promoted induction. For further induction by xenobiotics, slices were aged in 20 mM-aminopyrine or 8 mM-phenobarbital solutions instead of water for 42 h, or in a 25 mM-$MnCl_2$ solution for 72 h.

Tulip (*Tulipa forsteriana*, var. Yellow Impress) bulbs were submitted to the same treatments as artichoke tubers. Potato (*Solanum tuberosum*) and sweet potato (*Ipomea batatas*) tubers were sliced and aged for 24 h in distilled water in the dark. *Vicia sativa* (var. minor) seeds were germinated for 4 days before 48 h of aging in water or in a clofibrate (2 mM) emulsion in the dark. Seedlings were used after removal of the teguments. Wheat seeds (*Triticum aestivum* L., cv. Etoile de Choisy) were germinated for 48 h in the dark before another 48 h of aging in aerated distilled water in the presence or absence of 5 mM-phenobarbital. Radish (*Raphanus sativus*), castor bean (*Ricinus communis*), cucumber (*Cucurbita pepo*) and sunflower (*Helianthus annuus*) seedlings were grown on filter paper for 1 week in the dark before 24 h of aging in distilled water. Maize (*Zea mays* C., var. LG11) was germinated in moist vermiculite for 48 h; axes cut from the caryops were used as the source of microsomes. Ripe avocado (*Persea americana*) mesocarp was extracted using a Potter–Elvejhem homogenizer without any pretreatment. Bramble (*Rubus fruticosus*) microsomes were prepared from a cell suspension culture. *Mentha aquatica* L. and *Nasturtium officinale* R. B. were incubated for 3 days in aerated water in the dark, and the roots were removed before extraction.

Preparation of microsomes

All plant materials were homogenized at 4 °C in 100 mM-sodium phosphate buffer, pH 7.4, containing 15 mM-2-mercapto-

Abbreviations used: ECOD, 7-ethoxycoumarin *O*-de-ethylase; EROD, 7-ethoxyresorufin *O*-de-ethylase; ABT, 1-aminobenzotriazole; 11-DDNA, 11-dodecenoic acid; PBO, piperonyl butoxide; IC_{50}, inhibitor concn. causing 50 % inhibition.

ethanol, 1 mM-EDTA, 250 mM-sucrose and 40 mM-ascorbate. Addition of ascorbate to the extraction medium significantly (up to 100 %) improved the recovery of mono-oxygenase activities from most plant materials (the pH had to be kept at 7.4). Plant tissues were extracted using a Moulinex mixer or an Ultra-Turrax homogenizer. Cells were first disrupted by grinding in liquid N_2 in a mortar and then homogenized with a Moulinex mixer. Microsomes were then prepared by the procedure described previously (Werck-Reichart *et al.*, 1988) and stored at −20 °C. Limited loss of activity was observed after several months of storage, and significant activities could still be detected after 2 years.

Enzyme assays

The assay for ethoxycoumarin *O*-de-ethylase (ECOD) was adapted from the method of Ullrich & Weber (1972). The standard assay contained 250 μM-7-ethoxycoumarin (stock solution in dimethyl sulphoxide), 1 mM-glucose 6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase and 100–500 μg of microsomal protein in Tris/HCl (50 mM), pH 8.6. The temperature of the assay medium was allowed to equilibrate at 30 °C for 2 min in the thermostated cuvette before starting the assay by addition of 50 μM-NADPH. Fluorescence changes at 460 nm were directly recorded as a function of time, using an excitation wavelength of 380 nm. The assay was calibrated by addition of known amounts of 7-hydroxycoumarin to the incubation medium (5–50 pmol). Under these conditions, rates of 7-hydroxycoumarin formation were linear for 15–20 minutes.

The ethoxyresorufin *O*-de-ethylase (EROD) assay was adapted from the method of Burke & Mayer (1974). The standard assay contained 1.25 μM-7-ethoxyresorufin (dissolved in dimethyl sulphoxide), 1 mM-glucose 6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase and 100–250 μg of microsomal proteins in 2 ml of sodium phosphate (25 mM), pH 7.4. The temperature of the assay medium was equilibrated for 2 min at 30 °C before starting the assay by addition of 50 μM-NADPH. Activity was evaluated from the initial rate of fluorescence change (excitation 530 nm, emission 585 nm). Calibration was achieved by addition of known amounts of resorufin to the incubation medium (10–50 pmol). In most cases, the fluorescence increase was linear for 2–3 minutes.

To measure inhibition by CO and reversal of this inhibition by light, standard incubation mixtures were diluted in buffer previously saturated (by 2 min of bubbling) with CO. To achieve light reversion, the cuvette was removed from the fluorimeter and submitted for 2–3 min to white light from a 150 W heat filtered quartz lamp at 10 cm distance.

Other assays

Binding spectra with cytochrome *P*-450 were determined by differential spectroscopy using a Schimadzu MPS-2000 spectrophotometer. Various amounts of compound were added to diluted native oxidized microsomes (1–2 mg of proteins/ml). The absorbance change between 428 and 410 nm was related to the inhibitor concentration for determination of K_d.

Proteins were estimated by the method of Schacterle & Pollack (1973), using BSA as standard.

The data are means of duplicates (or triplicates for kinetic determinations); the data were fitted using a non-linear regression program written in Fortran and run on a DEC PDP/11 computer. The matrix solution delineated by Cleland (1983) was used.

RESULTS

Assay optimization

As shown in Table 1, EROD activity was much more dependent on temperature than was ECOD activity. Initial rates of both enzymic activities were highest at 30 °C or above. Above 30 °C, however, a significant loss of reaction linearity was observed. Therefore both assays were routinely run at 30 °C.

ECOD activity increased linearly with protein concentration up to 1 mg per assay; for EROD this value was 0.5 mg per assay. To optimize measurements, microsomal proteins were routinely used at around 250 μg per assay.

From Fig. 1 it appears that the two activities have completely different pH optima. ECOD activity was optimal at pH 8.6, whereas EROD, when measured at low ionic strength, showed a sharp optimum centered around pH 7.5.

Both ECOD and EROD were inhibited by high salt concentrations (Table 2). ECOD activity was optimal at very low ionic strength, between 0.05 and 0.1 mol/l, whereas EROD was slightly less sensitive, highest activities being measured at an ionic strength of around 0.15 mol/l.

Table 1. Temperature-dependence of ECOD and EROD activities

ECOD and EROD activities of Mn^{2+}-induced Jerusalem artichoke microsomes were measured as described in the Material and methods section directly in the thermostated cuvette. Buffer was prewarmed and temperature allowed to equilibrate for 5 min before addition of NADPH to start the reaction. Values are the means ± S.D. of two or three separate determinations. Activities are expressed as pkat/mg of microsomal protein. 'Linearity' indicates the length of time the reaction rate remained linear.

	ECOD		EROD	
Temperature (°C)	Activity (pkat/mg)	Linearity (min)	Activity (pkat/mg)	Linearity (min)
20	2.62 ± 0.05		0.76 ± 0.01	
25	2.79 ± 0.21		1.17 ± 0.02	8–10
30	3.13 ± 0.03	10–20	1.46 ± 0.03	2–5
35	3.14 ± 0.04		2.16 ± 0.07	< 2
40	2.90 ± 0.09	< 2	2.69 ± 0.13	< 1

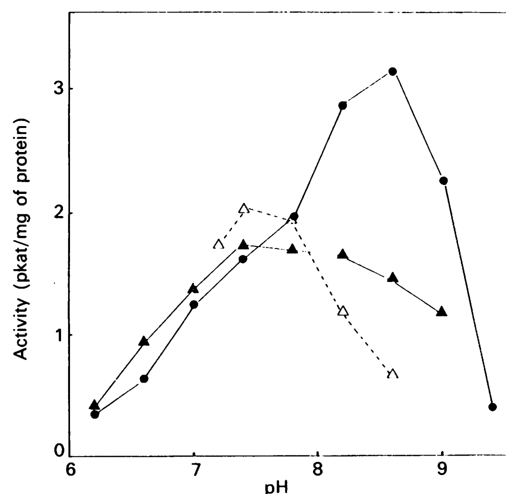

Fig. 1. pH dependence of ECOD and EROD activities

Values are the means ± S.D. of two or three separate determinations performed at 30 °C using Mn^{2+}-induced artichoke microsomes in a $KH_2PO_4/Na_2B_4O_3,10H_2O$ buffer mixture. ●, ECOD activity; ▲, EROD activity; △, EROD activity in Tris/HCl (50 mM).

Table 2. Effect of ionic strength on ECOD and EROD activities

Activities were determined in Mn^{2+}-induced Jerusalem artichoke microsomes at 30 °C in sodium phosphate buffer at pH 7.4 for EROD and pH 7.8 for ECOD. In an alternative set of experiments, ECOD activity was measured in Tris/HCl at pH 8.6. Values are means ± S.D. (n = 2 or 3). Activities are expressed in pkat/mg of protein.

Ionic strength (mol/l)	ECOD activity (pkat/mg)	EROD activity (pkat/mg)
Sodium phosphate		
0.524	0.80 ± 0.05	1.07 ± 0.02
0.262	1.41 ± 0.04	1.87 ± 0.03
0.131	2.21 ± 0.03	2.41 ± 0.23
0.065	2.61 ± 0.06	2.01 ± 0.02
Tris/HCl		
0.1	4.07 ± 0.09	
0.05	4.24 ± 0.03	
0.025	3.10 ± 0.05	

Dimethyl sulphoxide concentrations of up to 1.5% produced no inhibition of either activity. Stimulation of several O-deethylation reactions by Mg^{2+} has been described by Lubet *et al.* (1985). In our hands, $MgCl_2$ (≥ 2 mM) was inhibitory for both activities, probably due to increased ionic strength.

Cofactor requirements and kinetic properties

The kinetic properties of ECOD and EROD were studied in Mn^{2+}-induced artichoke microsomes as a function of substrate and NADPH concentrations (Table 3). Both reactions followed Michaelis–Menten kinetics. The apparent K_m values of ECOD were 161 ± 27 μM for ethoxycoumarin and 730 ± 110 nM for NADPH, with a maximal velocity of around 3 pkat/mg of protein. EROD had an apparent K_m of 400 ± 100 nM for ethoxyresorufin and of 2.76 ± 0.77 μM for NADPH. The $V_{max.}$ of EROD was slightly lower than that of ECOD, about 2.1 pkat/mg of protein.

ECOD and EROD showed an absolute requirement for reducing equivalents (Table 4). NADPH was much more efficient than NADH, especially in the case of ECOD, for which the maximal velocity catalysed by NADH never exceeded 4% of that observed in the presence of NADPH. The optimal NADH concentration (500 μM) allowed an EROD activity equivalent to 30% of the NADPH-dependent $V_{max.}$.

At non-saturating NADPH concentrations, an important synergistic effect of NADH was observed for both ECOD and EROD. Activities recorded when pyridine nucleotides were administered simultaneously were increased 1.5–2.5-fold as compared with the sum of activities measured when NADPH and NADH were given alone.

Table 3. Kinetic parameters of ECOD and EROD activities

Parameters were calculated as described in the Material and methods section from six duplicated measurements, using Mn^{2+}-induced artichoke microsomes as enzyme source.

Enzyme/substrate	K_m (μM)	$V_{max.}$ (pkat/mg of protein)
ECOD/ethoxycoumarin	161 ± 27	3.00 ± 0.22
ECOD/NADPH	0.73 ± 0.11	3.33 ± 0.12
EROD/ethoxyresorufin	0.40 ± 0.10	2.15 ± 0.15
EROD/NADPH	2.76 ± 0.77	2.09 ± 0.17

Table 4. Cofactor requirements of ECOD and EROD activities

Activities were measured in Mn^{2+}-induced artichoke microsomes in the presence of reduced pyridine nucleotide regeneration systems, as described in the Materials and methods section. Values are means ± S.D. of two separate measurements. 100% ECOD activity = 3.2 pkat/mg of protein; 100% EROD activity = 2.3 pkat/mg of protein.

	ECOD	
Cofactor	Relative activity (%)	Synergism (%)
None	0	
NADPH 100 μM	100 ± 9	
200 nM	25 ± 0	
100 nM	16 ± 0	
NADH 1 mM	3 ± 0.1	
500 μM	4 ± 0	
100 μM	3 ± 0.3	
50 μM	2 ± 0.1	
NADPH (100 nM) + NADH (100 μM)	48 ± 5	148
NADPH (200 nM) + NADH (100 μM)	48 ± 1	105

	EROD	
Cofactor	Relative activity (%)	Synergism (%)
None	0	
NADPH 100 μM	100 ± 3	
1 μM	22 ± 2	
NADH 500 μM	34 ± 2	
100 μM	32 ± 0.2	
50 μM	14 ± 1	
NADPH (1 μM) + NADH (50 μM)	90 ± 9	62
NADPH (1 μM) + NADH (100 μM)	85 ± 3	56

Involvement of cytochrome P-450

Results of experiments devised to test the cytochrome P-450 dependence of ECOD and EROD are shown in Table 5. Both reactions were inhibited by 70–90% in the presence of antibodies directed against artichoke NADPH–cytochrome c (P-450) reductase. CO inhibited ECOD as well as EROD by more than 50%. This inhibition was reversed by white light to 69% in the case of ECOD and to 48% in the case of EROD.

Metabolic inactivators of plant cytochromes P-450, i.e. ABT and 11-DDNA (Reichhart *et al.*, 1982; Salaün *et al.*, 1984) produced strong inhibition of ECOD and EROD activities. Respectively 66 and 62% of ECOD activity was lost after 5 min of preincubation in the presence of NADPH plus 100 μM-ABT or 100 μM-11-DDNA. A 1 mM concentration of either compound produced complete inactivation. In the case of EROD, about 50% inhibition was obtained with 100 μM-ABT or 100 μM-11-DDNA, but at 1 mM the inhibition did not exceed 77% with ABT or 85% with 11-DDNA.

Methylenedioxyphenyl compounds are catalytically oxidized into quasi-irreversible complexes with P-450 haem. They show inhibitory interactions with mammalian and insect cytochromes P-450 (Wilkinson *et al.*, 1984) and some, such as piperonyl butoxide (PBO), are used as insecticide synergists (Casida, 1970). In Jerusalem artichoke, PBO was a more efficient inhibitor

Table 5. Effects of cytochrome *P*-450 mono-oxygenase inhibitors on ECOD and EROD activities

Activities were determined in Mn^{2+}-induced artichoke microsomes. Inhibition by CO was measured as described in the Material and methods section. Rat monoclonal antibodies (150 μg of purified IgGs) directed against artichoke NADPH–cytochrome *c* reductase were preincubated for 5 min at 30 °C with microsomes before dilution into the assay medium. Monoclonal antibodies directed against an insect steroid transporter protein were used as an irrelevant control. The cytochrome *P*-450 suicide substrates ABT and 11-DDNA were preincubated for 5 min at 30 °C in the assay medium plus NADPH before addition of the substrate to initiate the reaction. PBO was added in the test without preincubation. Activities of control assays were 2.3 pkat/mg of microsomal protein in the case of ECOD and 1.8 pkat/mg of microsomal protein in the case of EROD. Values are means ± S.D. (n = 2–4). n.d., not determined.

Inhibitor	Relative activity (%)	
	ECOD	EROD
None	100.0 ± 0.5	100.0 ± 5.7
Anti-reductase IgGs	28.6 ± 2.7	11.8 ± 2.3
Irrelevant IgGs	96.8 ± 1.5	98.4 ± 0.1
CO/dark	46.5 ± 2.0	44.3 ± 4.4
CO/light	83.2 ± 7.7	70.8 ± 0.6
ABT 100 μM	33.9 ± 1.0	47.7 ± 2.0
1 mM	0	22.8 ± 1.5
11-DDNA 100 μM	38.0 ± 7.5	49.4 ± 3.7
1 mM	0	14.8 ± 1.5
PBO 5 μM	47.3 ± 0.8	71.1 ± 2.0
25 μM	29.0 ± 4.6	47.5 ± 3.4
Tetcyclasis 90 nM	n.d.	42.4 ± 2.8
180 nM	96.6 ± 12.0	24.7 ± 1.4
900 nM	31.4 ± 0	5.2 ± 0.5
1.8 μM	28.6 ± 1.3	4.1 ± 0.4

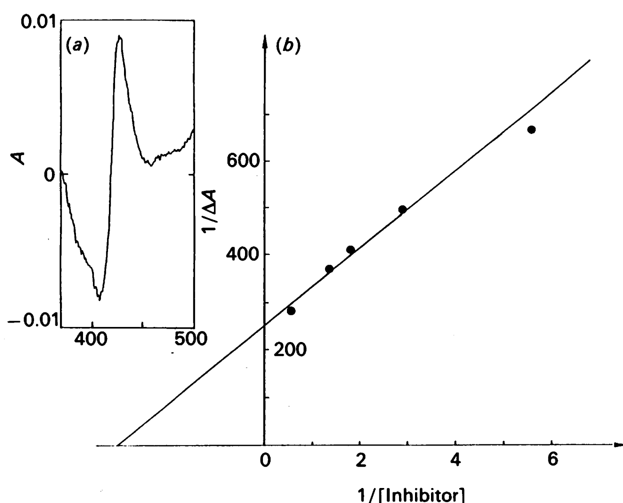

Fig. 2. Spectral changes induced by fixation of tetcyclasis on Mn^{2+}-induced artichoke microsomes

The base-line was recorded with native microsomes (2 mg/ml) in both cuvettes. Various concentrations of inhibitor dissolved in dimethyl sulphoxide were added to the sample cuvette, with identical volumes of dimethyl sulphoxide being added to the reference. (*a*) Binding spectrum is shown for saturating (10 μM) tetcyclasis concentration. (*b*) Double-reciprocal plot of the absorbance difference ($A_{428} - A_{410}$) versus inhibitor concentration.

of ECOD activity [IC_{50} (concn. causing 50 % inhibition) about 7.5 μM] than of EROD activity (IC_{50} about 25 μM).

Tetcyclasis belongs to the nitrogen-containing family of inhibitors that strongly bind to the ferric prosthetic haem iron of cytochrome *P*-450 and that are used as fungicides or plant growth regulators (Rademacher *et al.*, 1987). Intense type II binding spectra were elicited by addition of tetcyclasis to Jerusalem artichoke microsomes (Fig. 2), with a calculated K_d of about 330 nM. Table 5 shows that tetcyclasis is a very potent inhibitor of ECOD (IC_{50} of 600 nM) and, even more so, of EROD (IC_{50} of 75 nM) activity in Mn^{2+}-induced artichoke microsomes. In sharp contrast, inhibition of cinnamic acid or lauric acid hydroxylases in this material did not exceed 20 % even at inhibitor concentrations greater than 100 μM.

Cumene hydroperoxide-dependent reaction

Cumene hydroperoxide-dependent ECOD activity was recorded in induced artichoke microsomes. Cumene hydroperoxide (125 μM) sustained ECOD activities comparable with those observed with NADPH in aminopyrine-induced microsomes, but the linearity of the reaction did not continue for more than 1 min. The reaction did not proceed with boiled microsomes. At a lower (25 μM) cumene hydroperoxide concentration, activity was 3 times lower but remained linear for at least 5 min.

No cumene hydroperoxide-dependent EROD activity could be detected in any of the microsome preparations tested, including those which showed high cumene hydroperoxide-dependent ECOD activity and similar NADPH-catalysed ECOD and EROD activities.

Table 6. Distribution and induction of ECOD and EROD activities in higher plants

Activities were measured in microsomal fractions from plant tissues pretreated as described in the Material and methods section. Values are means of two or three measurements.

Plant species	Inducer	Activity (pkat/mg of protein)	
		ECOD	EROD
Jerusalem artichoke	None	0.09 ± 0.01	0
	Wounding	0.82 ± 0	0.31 ± 0.01
	$MnCl_2$	2.66 ± 0.10	1.69 ± 0.06
	Phenobarbital	1.60 ± 0.18	1.23 ± 0
	Aminopyrine	7.36 ± 0	7.41 ± 0.41
Tulip	None	0.05 ± 0	< 0.05
	Wounding	0.37 ± 0.02	0
	Phenobarbital	0.73 ± 0	0
Vicia	None	0.19 ± 0.01	0
	Clofibrate	0.45 ± 0.01	0
Wheat	None	0.15 ± 0.02	0
	Phenobarbital	1.57 ± 0.05	0.37 ± 0
Maize	None	0.12 ± 0.02	< 0.05
Sunflower	None	0.035	0
Castor bean	None	0.05	0
Radish	None	0.08	0
Cucumber	None	0.05	0
Avocado	None	1.57 ± 0.01	0.08 ± 0
Sweet potato	Wounding	0.06	0
Potato	Wounding	0.09	0
Watercress	None	0.09	< 0.05
Wild mint	None	0.10	< 0.05
Bramble	None	0.04	0

Distribution and inducibility of O-dealkylating activities in higher plants

Table 6 shows that low but significant ECOD activity was detected in microsomes from most non-induced plant tissues. These include quiescent Jerusalem artichoke tuber, *Vicia*, wheat, radish, cucumber, castor bean, sunflower seedlings, maize embryos, tulip bulb, ripe avocado mesocarp, etiolated mint and watercress, and bramble cells. Highest activities were measured in avocado mesocarp and in young seedlings from maize, wheat and *Vicia*.

These basal ECOD activities were increased by wounding, by up to 10-fold in artichoke and 7.4-fold in tulip bulb, but the greatest stimulations were observed after aging in the presence of chemical inducers. Aging in the presence of 8 mM-phenobarbital produced a doubling of ECOD activity, as compared with aging on water, in artichoke and tulip, and 5 mM-phenobarbital produced a 10-fold increase in the basal ECOD activity in young wheat shoots. Mn^{2+} ions and especially aminopyrine were the best inducers of ECOD activity in artichoke tuber, producing respectively 3.2-fold and 9-fold increases in activity as compared with the wounded tissues. A doubling of ECOD activity due to clofibrate was also observed in *Vicia* seedlings.

EROD activity was too low to be reliably measured in all non-induced plant microsomes tested, except in avocado mesocarp, which contained low but measurable activity. However, after induction by wounding and/or chemicals, high EROD activities were observed in artichoke and wheat. Aminopyrine was the best inducer of EROD activity in the artichoke tuber, producing a 24-fold increase in EROD activity as compared with the wounded tissues, but this inducer was not tested on the other plants.

Cross-inhibition by other substrates of plant cytochromes P-450

Together, ECOD and EROD bring to six the number of cytochrome P-450-dependent mono-oxygenase reactions that we have demonstrated in aminopyrine-induced artichoke microsomes. In order to test whether some of these activities could be catalysed by the same *P*-450 isoform, we examined the ability of various demonstrated and also putative substrates to inhibit ECOD and EROD activities of artichoke microsomes.

Cinnamic acid and lauric acid are hydroxylated, and chlorotoluron and aminopyrine are *N*-demethylated, by aminopyrine-induced cytochrome *P*-450 in artichoke microsomes (Fonné, 1985; Fonné-Pfister *et al.*, 1988). The herbicide diclofop is hydroxylated by cytochrome *P*-450 in wheat microsomes (Zimmerlin & Durst, 1990) and binds to artichoke microsomes, producing a type I interaction spectrum. Chlorsulfuron and ioxynil were included in this study as other possible substrates.

Except chlorsulfuron, most of the compounds tested produced low but significant inhibition of ECOD activity (Table 7). This inhibition did not exceed 25%, even at concentrations higher than 10 times the apparent K_m, with cinnamic acid, lauric acid and diclofop. In contrast, at concentrations equal to their apparent K_m values for artichoke cytochrome *P*-450, aminopyrine and chlorotoluron produced an inhibition of more than 50%.

A different inhibition pattern was observed in the case of EROD (Table 8). Cinnamic acid, lauric acid and chlorsulfuron produced no inhibition, or even a slight stimulation, of EROD activity. On the contrary, chlorotoluron, aminopyrine, diclofop and ioxynil were more potent inhibitors of EROD than of ECOD activity. The most efficient were chlorotoluron and ioxynil, which reduced the activity by more than 80% at 100 μM, whereas 100 μM-diclofop or -7-ethoxycoumarin led to inhibition of between 40 and 50%.

Table 7. Sensitivity of ECOD activity to inhibition by demonstrated or possible substrates of plant cytochromes P-450

ECOD activities were measured in aminopyrine-induced artichoke microsomes in the presence of 250 μM-7-ethoxycoumarin. When inhibitors were dissolved in organic solvents the activity was related to the activity observed with the solvent alone. When possible, dimethyl sulphoxide which produces little or no alteration of the activity, was used as a solvent. 100% activity = 3.86 ± 0 pkat/mg of proteins. Values are means ± S.D. of two measurements. Apparent K_m values are those described for the particular inhibitor for plant cytochrome *P*-450.

Inhibitor		Relative activity of ECOD (%)	Apparent K_m
None		100	
Cinnamic acid	15 μM	94.8 ± 7.4	2 μM
	50 μM	86.9 ± 6.5	
	100 μM	79.9 ± 7.4	
Lauric acid	25 μM	76.4 ± 3.9	9 μM
	100 μM	79.0 ± 3.5	
Chlorotoluron	100 μM	86.9 ± 1.7	500 μM
	250 μM	58.9 ± 5.4	
Aminopyrine	5 mM	59.7 ± 2.9	9 mM
	10 mM	48.5 ± 1.6	
7-Ethoxyresorufin	1.25 μM	83.9 ± 3.6	400 nM
	3 μM	64.9 ± 0.8	
Diclofop	100 μM	82.1 ± 6.1	9 μM
	200 μM	76.0	
Chlorsulfuron	100 μM	93.4 ± 1.4	
	250 μM	93.2 ± 0	
Ioxynil	100 μM	71.0 ± 2.8	

Table 8. Sensitivity of EROD activity to inhibition by demonstrated or possible substrates of plant cytochromes P-450

EROD activities were measured in aminopyrine-induced artichoke microsomes in the presence of 1.25 μM-7-ethoxyresorufin; 100% activity = 3.88 ± 0.16 pkat/mg of protein. Values are means ± S.D. of two measurements. Apparent K_m values are as described in the legend to Table 7.

Inhibitor		Relative activity of EROD (%)	Apparent K_m
None		100	
Cinnamic acid	15 μM	109.5 ± 6.0	2 μM
	50 μM	116.8 ± 3.5	
	100 μM	115.0 ± 5.3	
Lauric acid	25 μM	114.1 ± 7.1	9 μM
	100 μM	97.0 ± 2.9	
Chlorotoluron	12 μM	51.9 ± 1.5	500 μM
	25 μM	37.5 ± 0.1	
	50 μM	26.5 ± 2.2	
	100 μM	16.7 ± 0.5	
Aminopyrine	5 mM	41.0 ± 1.5	9 mM
	10 mM	21.9 ± 3.6	
7-Ethoxycoumarin	50 μM	61.9 ± 0.9	160 μM
	100 μM	41.6 ± 0.9	
	200 μM	22.6 ± 2.2	
Diclofop	100 μM	48.1 ± 0.3	9 μM
	250 μM	29.2 ± 1.6	
	500 μM	5.9 ± 0.4	
Chlorsulfuron	100 μM	105.9 ± 0.1	
	250 μM	80.0 ± 0.7	
Ioxynil	100 μM	19.1 ± 4.5	

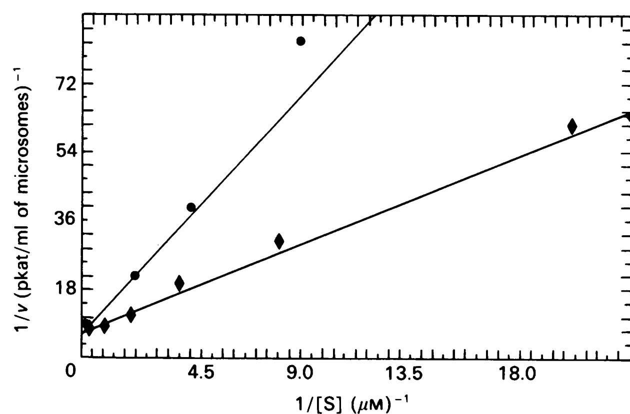

Fig. 3. Lineweaver–Burk plot of inhibition of EROD by 7-ethoxycoumarin

Activities were determined in aminopyrine-induced microsomes in conditions shown to be optimal for EROD activity (see the Material and methods section). ●, Plus 100 μM-7-ethoxycoumarin; ◆, without inhibitor.

A concentration of 7-ethoxycoumarin equivalent to its apparent K_m for ECOD inhibited over 50% of EROD activity. Data in Fig. 3 show that 7-ethoxycoumarin is a competitive inhibitor of EROD activity, with a K_i of 59 μM.

Coumarin hydroxylase activity

To detect a putative microsomal coumarin 7-hydroxylase, coumarin was assayed as a substrate of microsomes from artichoke, tulip, wheat and *Vicia* tissues exposed to various inducers. Using the same assay as for ECOD determination, no hydroxycoumarin formation could be measured.

DISCUSSION

The results described in this paper show that plant microsomes are able to *O*-dealkylate 7-ethoxycoumarin as well as 7-ethoxyresorufin. As in animal tissues, both reactions are catalysed by cytochrome *P*-450. This is clearly shown (1) by the dependence of the activities on reducing equivalents and their inhibition by antibodies directed against NADPH–cytochrome *c* (*P*-450) reductase; (2) by their light-reversible inhibition by CO; (3) by the synergistic effect of NADH observed at limiting NADPH concentrations; (4) by the inactivation produced by the mechanism-based inhibitors ABT, 11-DDNA and PBO; and (5) by the potent inhibition produced by the norbonanodiazetine derivative tetcyclasis.

Moreover, our results unambiguously demonstrate that two independent cytochrome *P*-450 isoforms are involved in ethoxycoumarin and ethoxyresorufin de-ethylations. Supportive evidence includes the different physical and kinetic properties of the two reactions, the dissimilarities observed in their sensitivities toward inhibitors and chemical inducers, and in their distributions among plant species.

The two activities exhibited different sensitivities to temperature and completely different pH optima (pH 8.6 for ECOD and pH 7.4 for EROD). These optimal pH values are also significantly different from the pH optima described for the same reactions in mammalian liver (Prough *et al.*, 1978). At the same time, artichoke ECOD and EROD exhibited slightly different sensitivities toward ionic strength; however, both were inhibited by high salt concentrations ($I > 0.15$ mol/l). This is markedly different from ECOD and EROD from animal sources, which are usually measured in presence of high salt ($MgCl_2$ and KCl) concentrations.

NADPH was by far the most efficient electron donor for both reactions, but ECOD displayed a lower apparent K_m for NADPH (730 nM) than did EROD (2.76 μM). It is noteworthy that both values are significantly lower than the K_m for NADPH of other cytochrome *P*-450-catalysed reactions in artichoke tubers (Benveniste *et al.*, 1977; Salaün, 1985; Fonné, 1985) and lower than the K_m of the purified artichoke NADPH–cytochrome *c* (*P*-450) reductase (20 μM; Benveniste *et al.*, 1986). Data currently available do not afford an explanation for these discrepancies. On the other hand, NADH supported a significant turnover of EROD activity (up to 35% of the NADPH-dependent activity), but produced only less than 4% of NADPH-sustained ECOD activity.

Cumene hydroperoxide-supported 7-ethoxycoumarin and 7-ethoxyresorufin *o*-dealkylations have been reported in rat liver microsomes (Burke & Mayer, 1975; Rahimtula & O'Brien, 1975). In artichoke microsomes, however, cumene hydroperoxide exclusively sustained ECOD activity. Repeated attempts to detect cumene hydroperoxide-dependent EROD or cinnamic acid hydroxylase in induced artichoke or *Vicia* microsomes were unsuccessful.

From the results shown in Table 6, it appears likely that 7-ethoxycoumarin de-ethylation is catalysed by a constitutive form of cytochrome *P*-450. Low levels of ECOD activity were detected in most non-induced plant microsomes tested (except microsomes prepared from some aquatic plants, i.e. *Wolfia* or *Elodea*). This basal ECOD activity was greatly enhanced by various *P*-450-inducing treatments. Wounding resulted in a 5–10-fold increase in the basal activity, and chemical inducers, Mn^{2+}, phenobarbital, aminopyrine or clofibrate produced a further 2–10-fold elevation of activity in the wounded tissues. Aminopyrine was the most efficient chemical inducer of ECOD as well as EROD activity in Jerusalem artichoke, 20 mM-aminopyrine producing respectively 9- and 24-fold increases in ECOD and EROD activities.

A study of the distribution of EROD among higher plants showed that this activity was absent, or too low to be detectable, in non-induced tissues from the plants that we tested. Wounding or chemical treatment resulted in the appearance of significant activity in some plant species such as artichoke and wheat, whereas in other plants such as tulip and *Vicia* there was no response to any inducing treatment. It is noteworthy that these include plants whose ECOD activity could be efficiently stimulated.

At first sight, avocado seems to be an exception, since we observed significant EROD activity in microsomes prepared from untreated mesocarp. It must however be stressed that levels of ECOD and EROD activities varied greatly from one microsomal preparation to another, some preparations being devoid of ECOD and EROD activity while still able to hydroxylate cinnamic and lauric acids. Induction due to various pre- or post-harvest treatments of the avocado pears from different origins could account for these observations.

Further arguments in favour of different cytochrome *P*-450 isoforms were given by inhibition studies. ECOD appeared to be more susceptible to inhibition by mechanism-based inhibitors such as ABT, 11-DDNA or PBO or by 'physiological' substrates such as cinnamic or lauric acid. On the other hand, EROD activity was strongly inhibited by most of the phytosanitary compounds included in this study, i.e. chlorotoluron, ioxynil, diclofop and tetcyclasis. No ioxynil or diclofop metabolism by artichoke microsomes could be detected. Chlorotoluron is however actively *N*-demethylated by artichoke microsomes (Fonné, 1985). The possibility that chlorotoluron and 7-ethoxyresorufin could be dealkylated by a single *P*-450 isoform thus has to be considered in future experiments. Tetcyclasis is an extremely potent inhibitor of EROD activity, producing 50% inhibition at concentrations of less than 0.1 μM. Such concentrations are of

the same order of magnitude as the concentrations described to be inhibitory for the ent-kaurene oxidation steps of the gibberellin pathway (Rademacher *et al.*, 1987) or for chlorotoluron *N*-demethylation in cotton cells (Cole & Owen, 1987). Tetcyclasis concentrations needed to inhibit other plant cytochrome *P*-450-dependent mono-oxygenases, including the *P*-450 isoform involved in sterol biosynthesis (10 μM; Taton *et al.*, 1988), are significantly higher.

It is clear that two independent cytochromes *P*-450 are involved in 7-ethoxycoumarin and 7-ethoxyresorufin dealkylation reactions in plants. Moreover, competition experiments comparing sensitivities toward tetcyclasis, plant distribution and induction patterns (cf. Fonné-Pfister *et al.*, 1988; Salaun *et al.*, 1982) leave little doubt that these two cytochrome isoforms are different from the enzymes involved in cinnamic acid and lauric acid hydroxylation. However, further experiments will be needed to unambiguously differentiate ECOD and EROD catalysing isoforms from the enzymes responsible for the metabolism of aminopyrine and chlorotoluron. Furthermore, as 7-ethoxycoumarin is an efficient competitive inhibitor of EROD activity, the possibility that the EROD-catalysing isoform could contribute to the metabolism of ethoxycoumarin in some plant microsomes will need further consideration.

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