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Epoxidation of *cis* and *trans* Δ^9-unsaturated lauric acids by a cytochrome P-450-dependent system from higher plant microsomes

Jean-Pierre Salaün, Daniel Weissbart, Francis Durst, Philippe Pflieger* and Charles Mioskowski*

Laboratoire d'Enzymologie Cellulaire et Moléculaire, Université Louis Pasteur – CNRS UA 1182, 28, rue Goethe, F-67083 Strasbourg Cedex and **Laboratoire de Chimie Bio-organique, Université Louis Pasteur – CNRS UA 31-Faculté de Pharmacie, 74 route du Rhin, F-67048 Strasbourg Cedex, France*

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A new epoxygenase, differing in substrate and stereospecificity from those previously described in mammals, bacteria and *Vicia faba*, has been characterized in higher plants. A microsomal fraction from Jerusalem artichoke tubers which catalyses in-chain monohydroxylation of lauric acid has now been found to convert synthetic 9-dodecenoic acid (9-DDNA) to 9,10-epoxylauric acid. Epoxidation of the *cis* and *trans* isomers proceeds at similar rates affording the corresponding *cis* and *trans* epoxides. This reaction is dependent upon O_2 and NADPH and does not occur with boiled microsomes. Lauric acid, carbon monoxide and polyclonal antibody against NADPH-cytochrome P-450 reductase from Jerusalem artichoke inhibit epoxide formation. Compared to freshly sliced tuber tissues, epoxidation activity was strongly enhanced in tissues aged in 20 mM aminopyrine solution. The possibility that 9,10-epoxidation of 9-DDNA is catalyzed by the cytochrome P-450 isoenzyme involved in lauric acid in-chain hydroxylation in this plant was investigated.

Cytochrome P-450; Epoxidation; Hydroxylation; Fatty acid; Induction; (*Helianthus tuberosus*)

1. INTRODUCTION

Mammalian monooxygenases catalyze various epoxidation reactions of essential fatty acids and fatty acid derivatives [1–4]. These oxidized products are implicated in the biosynthetic pathway of cellular mediators such as prostaglandins or leukotrienes.

The bacterial system described by Fulco and coworkers [5,6] catalyses monohydroxylation at the ω-1, ω-2 and ω-3 positions of palmitoleic acid ($C16:1^{\Delta 9}$) or epoxidation at the C9-C10 position. Monohydroxypalmitoleic acids are very poor substrates for epoxidation. *trans*-9-Hexadecenoate is 60% less effective as a substrate for epoxidation than the *cis* isomer but not for in-chain hydroxylation. Experimental evidence strongly supports the concept that both hydroxylation and epoxidation are catalysed by an identical soluble P-450 complex. This phenobarbital-inducible enzyme has been characterized [7].

Recently, it has been reported that a fungal cytochrome P-450 monooxygenase also catalysed subterminal hydroxylation (ω-1, ω-2 and ω-3) of laurate and palmitate [8].

Oxygenated fatty acids take an important place in secondary plant metabolism [9]. Enzyme preparations capable of epoxidizing an unsaturated fatty acid were first described by Croteau and Kollattukudy [10]. The plant enzyme catalyzing epoxidation of 18-hydroxy-*cis*-9-octadecenoic acid required ATP, CoA, NADPH and O_2 for maximal activity at pH 9.0. The epoxidase, which is located in a 3000 × *g* particulate fraction from young spinach leaves, has a stringent substrate specificity. Neither oleic acid nor 18-hydroxyelaidic acid (*trans* isomer of the substrate) was epoxidized by the fraction. This uncommon epoxidase, observed in no other living organisms and

Correspondence address: J.-P. Salaün, Laboratoire d'Enzymologie Cellulaire et Moléculaire, Université Louis Pasteur – CNRS UA 1182, 28, rue Goethe, F-67083 Strasbourg Cedex, France

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involved in the biosynthesis of plant cuticle, is the only example of a plant epoxidase described thus far.

Using lauric acid as a model substrate for P-450-dependent hydroxylation of fatty acids, we showed previously that two different P-450 enzymes, never present in the same plant, hydroxylate this fatty acid [11]. An in-chain hydroxylating enzyme (IC-LAH), first discovered in the microsomal fraction from Jerusalem artichoke tubers, catalyzes hydroxylation principally at the C9 position [12,13]. On the other hand, a C12 hydoxylase (ω-LAH) has been characterized in microsomal fractions from various Leguminosae [14].

To gain better insights into the mechanisms of fatty acid oxygenation in plants, we synthesized unsaturated analogs of lauric acid and used them to probe the ability of plant cytochrome P-450 to oxidize aliphatic sp^2 carbons. Here, we describe the epoxidation of 9-DDNA by microsomes from aminopyrine-induced Jerusalem artichoke tuber tissues.

2. MATERIALS AND METHODS

2.1. *Materials*

Lauric acid, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase and aminopyrine were purchased from Sigma; acetonitrile from BDH; [1-^{14}C]lauric acid and Na^{14}CN from CEA. 9-[1-^{14}C]Dodecenoic acids were synthesized in to by a known procedure summarized below.

2.2. *Plant materials*

Jerusalem artichoke tubers (*Helianthus tuberosus* L., CV. Blanc commun) grown in the facilities of the University Louis Pasteur were stored in polyethylene bags at 4°C in the dark. Tubers were sliced (1 mm thick, 100 g fresh wt), rinsed with tap water for 30 min to wash off the polyphenols produced by wounding, then with distilled water. Approx. 100 g fresh wet of tuber slices were incubated for 48 h, in the dark at room temperature, in 2-l Erlenmeyer flasks containing 1.5 l distilled water plus 20 mM aminopyrine. The aging solutions were bubbled continuously with a filtered, moist stream of air (4.5 l/min).

2.3. *Microsomal preparations*

Microsomal pellets were prepared by homogenizing 100 g of tuber slices at 4°C with an Ultra Turrax in 100 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 15 mM 2-mercaptoethanol and 10% (v/v) glycerol. The homogenate was filtered through cheesecloth and centrifuged for 10 min at 10000 × *g*. The supernatant was centrifuged for 60 min at 100000 × *g*. The resulting pellets were pooled, washed and centrifuged in the same buffer for 60 min at 100000 × *g*. The final pellets were resuspended in 10 ml of 0.1 M sodium phosphate (pH 7.4), containing 1.5 mM 2-mercaptoethanol and 30% (v/v) glycerol and stored at −20°C without appreciable loss of enzyme activity for several weeks.

2.4. *Measurement of enzyme activities*

In-chain lauric acid hydroxylase was assayed as in [14]. The standard assay mixture contained, in a final volume of 0.2 ml, 0.1–1 mg microsomal proteins, 0.1 M sodium phosphate buffer (pH 7.4), 1 mM NADPH, 6.7 mM glucose 6-phosphate, 0.4 U glucose-6-phosphate dehydrogenase, 2.12 μM [1-^{14}C]lauric acid (59 Ci/mol) and sodium laurate to make the final concentration 100 μM. The reaction was stopped after 20 min incubation at 30°C by adding 200 μl acetonitrile. After 10 min in cooling on ice and rapid centrifugation (10 min at 3000 rpm) aliquots (50–100 μl) of the supernatant were analysed by RP-HPLC or TLC. Oxidation of 9-DDNA was determined via the same procedure using 9-[1-^{14}C]dodecenoic acid (17.7 Ci/mol) as substrate. All experiments were performed with a synthetic mixture of 9-DDNA (*cis/trans* = 80:20) except in studies on metabolism of each form. 9-DDNA concentrations were at least 3-fold the K_m value.

2.5. *Synthesis of 9-[1-^{14}C]dodecenoic and epoxides of 9-[1-^{14}C]dodecenoic acids*

Radiolabelled compounds were synthesized according to established procedures.

9-[1-^{14}C]Dodecenoic acid was obtained in five steps starting from 1-bromo-8-octanol [15]. Treatment of the corresponding triphenylphosphonium bromide with lithium diisopropylamide followed by the addition of propanal gave 8-undecenol, which after bromination, labelling with Na^{14}CN (spec. act. 17.7 Ci/mol) and saponification led to 9-dodecenoic acid (80:20 mixture of *cis* and *trans* isomers).

The corresponding epoxides (*cis/trans* = 80:20) were obtained using *m*-chloroperbenzoic acid. All reaction products gave satisfactory ^{1}H NMR (200 MHz) and IR analysis.

2.6. *Chromatographic conditions*

Reverse-phase high-pressure liquid chromatography (RP-HPLC) was performed as described [14] with minor modifications. For analysis of radioactivity distribution, reaction products were injected either as 50 μl methanol extract or directly as 100 μl reaction medium containing 50% acetonitrile. Samples were developed (2 ml/min) using an initial mobile phase of 25:75:0.2 (by vol.) acetonitrile/water/acetic acid. A linear gradient (0–100%) of 80% acetonitrile in aqueous acetic acid was applied 40 min after injection at the same flow rate for 25 min.

Purification of the *cis* and *trans* isomers of synthetic 9-dodecenoic acid (*cis/trans* = 80:20) by RP-HPLC was carried out using an isocratic mobile phase (2 ml/min) composed of 45:55:0.2 (v/v) acetonitrile/water/acetic acid. Radioactive effluents were collected and compounds extracted twice with 1:1 (v/v) benzene/diethyl ether. This purification procedure was also used with the appropriate mobile phase before GC-MS analysis of reaction products. Radioactivity of RP-HPLC effluents was monitored with a computerized on-line solid scintillation counter (Ramona-D Isomess, FRG).

For quantitative evaluation, 100-μl aliquots of the incubation medium were spotted onto silica TLC plates. The plates were

developed in a system of 70:30:1 (v/v) diethyl ether/light petroleum (b.p. 40–60°C)/formic acid. After detection of polar reaction products with a thin-layer scanner (Berthold LB 2723), the areas corresponding to lauric acid (R_f 0.85), 9-dodecenoic acid (R_f 0.84), *cis*- and *trans*-9,10-epoxylaurates (R_f 0.69), 8-, 9- and 10-hydroxylaurates (as a bulk, R_f 0.54), were scraped into counting vials and the radioactivity measured with an Intertechnique SL 4000 liquid scintillation counter.

2.7. *Inhibition studies*

The effect of anti-reductase antibody was measured by incubating microsomes and lauric acid or 9-DDNA with rabbit polyclonal antibodies (5.5 mg protein/ml) raised against purified NADPH-cytochrome P-450 reductase from Jerusalem artichoke tubers [16].

Enzyme inactivation was measured by incubating 9-decenoic acid (added in 2 μl of 50% aqueous ethanol) and NADPH with the same microsomal fraction in the absence of radiolabelled laurate or 9-dodecenoate at 25°C for various periods of time. An equal amount of ethanol was added to the control incubation. After the desired incubation period, the radiolabelled substrates were added to the mixture and the incubation allowed to continue for a further 5 min.

To measure CO inhibition, reaction vials containing the complete system were prepared on ice and bubbled for 15 min with a 1.25:1:4 $CO/O_2/N_2$ gas mixture. The vials were transferred to a water bath and incubated for 20 min at 30°C either in darkness or under illumination with white light from a 150 W heat-filtered quartz lamp at a distance of 15 cm.

2.8. *Gas chromatography-mass spectrometry*

Products formed were first purified by RP-HPLC as described above. An ethereal solution of diazomethane was used for methylation. The instrument was a Nermag coupled to a Varian 6000 gas chromatograph. Samples were run on an SE 30 capillary column with temperature programming from 80°C at 4 degree/min. Spectra were recorded in the electron impact mode with an electron energy of 70 eV. Fragmentation patterns were compared to those of authentic *cis*- and *trans*-9,10-epoxylauric acids.

2.9. *Spectroscopic assays*

Spectrophotometric hemoprotein determinations were carried out on a Shimadzu SP 2000 instrument. Cytochromes P-450 and b_5 were measured as described by Omura and Sato [17] using the following extinction coefficients: 91 $mM^{-1} \cdot cm^{-1}$ for the 450–490 nm absorbance difference, and 185 $mM^{-1} \cdot cm^{-1}$ for the 424–409 nm absorbance difference respectively. Microsomal protein was estimated by the method of Schackterle and Pollack [18].

3. RESULTS

3.1. *Incubation of microsomes with lauric acid and 9-dodecenoic acid*

We showed previously that IC-LAH from Jerusalem artichoke microsomes was strongly enhanced when tuber slices were aged in aqueous aminopyrine solution [19]. The proportion of each monohydroxylaurate formed as a percentage of the bulk was calculated from 10 separate experiments to be 27 ± 2.5, 62 ± 2.7 and 11 ± 0.9% for 10-, 9- and 8-hydroxylaurate, respectively. These results, which are similar to those obtained with non-induced tissues, are illustrated in fig.1A (peaks d–f, respectively). When microsomes were incubated with the 80:20 synthetic mixture of *cis*- and *trans*-9-[1-^{14}C]dodecenoic acids, NADPH and air, major radioactive metabolites that were less polar than the in-chain hydroxylaurates (fig.1B) were formed. These compounds (peaks h,i) showed the same TLC mobility and the same retention time in GC and RP-HPLC as synthetic 9,10-[1-^{14}C]epoxylaurates. Moreover, GC-MS analysis of the methyl ester derivatives of the synthetic references and reaction products formed demonstrated identical fragmentation patterns on EI mass spectral analysis (not shown). The products associated with peaks h and i correspond to *cis*- and *trans*-9,10-epoxylauric acids, respectively. The percentages of *cis* (78%) and *trans* (22%) isomers formed are close to those calculated for the synthetic substrate. The more polar but minor metabolites (figs 1,2, peaks a,b) have not been characterized but are suspected to be diols formed from 9,10-epoxylaurates. Peak c (figs 1,2) contains 8-hydroxy-9-dodecenoic acid, since it yielded 8-hydroxylauric acid plus an unknown metabolite after hydrogenation. From kinetic studies, the apparent K_m of the reaction was estimated as 24.0 μM and the V_{max} as 250 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein. Epoxidation proceeds linearly up to 20 min for protein concentrations below 380 μg at 25°C.

The fact that lauric acid (not shown) inhibited both epoxide and 8-hydroxy-9-dodecenoate formation (apparent K_i = 11 μM) and that, conversely, 9-DDNA inhibited laurate hydroxylation (K_i = 9.4 μM), prompted us to perform microsomal incubations with radioactive laurate and 9-dodecenoate together. As shown in fig.1C, lauric acid and 9-dodecenoic acid were concurrently metabolized by the microsomal fraction and the different reaction products were well resolved by RP-HPLC. Equal amounts of polar products were formed by microsomes incubated with similar concentrations of the different substrates: 119 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein were formed from 124.5 μM laurate (fig.1A, peaks d,e,f), 133 $pmol \cdot min^{-1} \cdot mg^{-1}$ protein from 146 μM 9-DDNA (fig.1B, peaks h,i) and

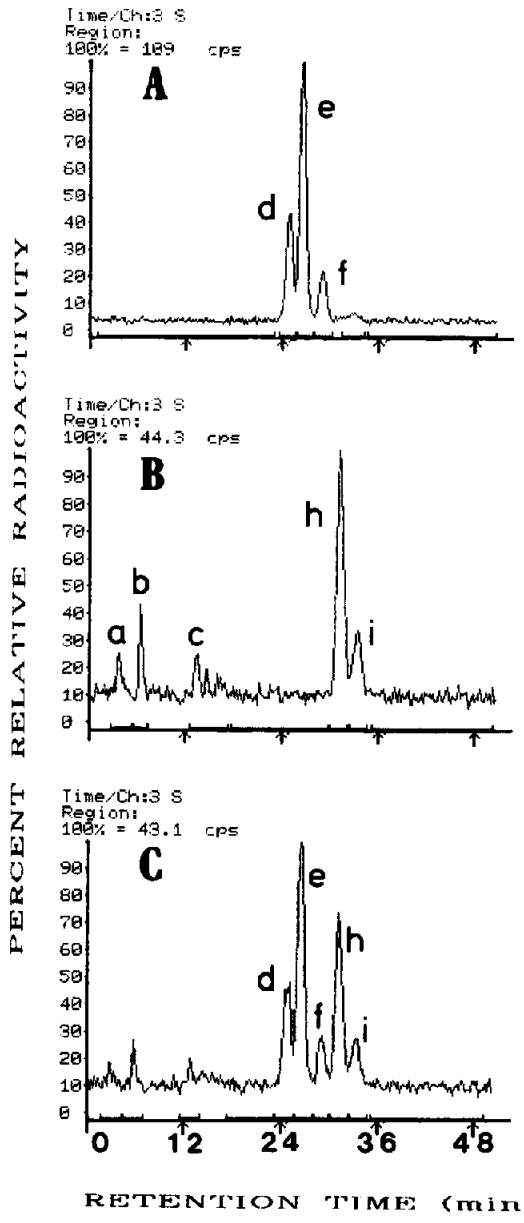


Fig.1. RP-HPLC elution profiles for incubation of microsomes with radiolabelled fatty acids using a mixture of $CH_3CN/H_2O/CH_3COOH$ (25:75:0.2, v/v) as mobile phase at a flow rate of 2 ml/min. Incubations with lauric acid (A), 9-DDNA (B) and lauric acid plus 9-DDNA (C) produced oxygenated metabolites which were: (a,b) diol derivatives (the products formed have not been fully characterized), (c) 8-hydroxy-9-dodecenoate, (d) 10-hydroxylaurate, (e) 9-hydroxylaurate, (f) 8-hydroxylaurate, (h) *cis*-9,10-epoxylaurate and (i) *trans*-9,10-epoxylaurate. For 9-DDNA, the proportion of each isomer in the synthesized substrate was 80% *cis* and 20% *trans*.

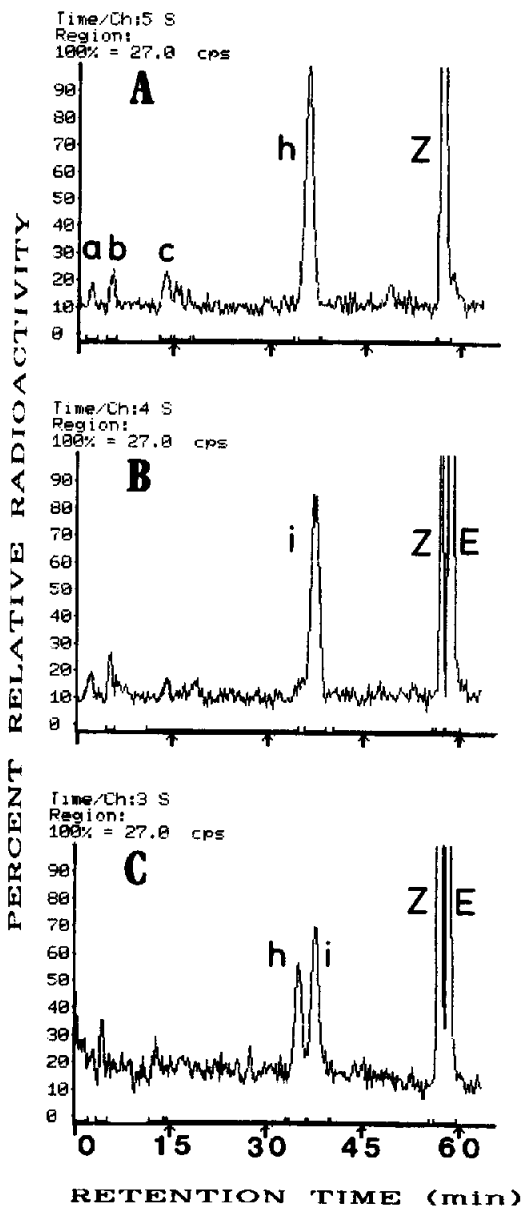


Fig.2. RP-HPLC elution profiles for incubation of microsomes with radiolabelled 9-DDNA using first a mixture of $CH_3CN/H_2O/CH_3COOH$ (25:75:0.2, v/v). After 40 min a linear gradient of 80% acetonitrile in aqueous acetic acid was applied at a flow rate of 2 ml/min. Incubations with *cis*-9-DDNA (*cis/trans* = 98:2) (A), *trans*-9-DDNA (*cis/trans* = 10:90) (B) and an equimolar mixture of *cis*- and *trans*-9-DDNA (*cis/trans* = 48:52) (C) produced oxygenated metabolites which were: (h) *cis*-9,10-epoxylaurate and (i) *trans*-9,10-epoxylaurate. Residual substrates were present in peaks (Z) *cis*-9-DDNA and (E) *trans*-9-DDNA.

138 pmol·min^{-1}·mg^{-1} protein from 135.5 μM of an equimolar mixture of laurate and 9-DDNA (fig.1C, peaks d,e,f,h,i).

3.2. *Metabolism of* cis- *and* trans-*9-dodecenoic acids by microsomes*

To confirm the unusual epoxidation of the *trans* isomer, we purified the two isomeric forms of 9-dodecenoic acid using RP-HPLC as described in section 2.6. The two radiolabelled isomers were incubated either alone or as an almost equimolar mixture (48% *cis* and 52% *trans*) with artichoke microsomes and cofactors. The RP-HPLC elution profiles of radiolabelled metabolites are shown in fig.2. When the *cis* or *trans* isomer was incubated alone (fig.2A and B, respectively), a major radioactive metabolite was formed in each case. Their retention times correspond to *cis*-9,10-epoxylaurate (fig.2A, peak h) and *trans*-9,10-epoxylaurate (fig.2B, peak i). The rates of formation of both compounds were very similar. This was confirmed by incubating both isomers together as shown in fig.2C (peaks h,i). The enzyme activities in fig.2A–C amounted to 187, 181 and 76 pmol·min^{-1}·mg^{-1} protein with substrate concentrations of 114, 84 and 69 μM, respectively. These experiments confirm the capacity of microsomes from aminopyrine-induced tuber tissues to catalyze 9,10-epoxidation of *cis*- as well as *trans*-9-dodecenoic acid.

3.3. *Effect of incubation conditions on epoxidation of 9-dodecenoate and hydroxylation of laurate*

As previously shown for P-450-catalysed hydroxylation of lauric acid, both molecular oxygen and NADPH were absolute requirements for epoxidation of 9-DDNA (table 1). NADH could only partly substitute for NADPH in either reaction. Further confirmation of the involvement of NADPH-cytochrome P-450 reductase in 9-DDNA epoxidation was provided by the strong inhibitory effect of polyclonal antibodies (IgG) raised against NADPH-cytochrome P-450 reductase purified from artichoke tuber tissues [16].

CO (table 2), which interacts with the active site of cytochrome P-450 enzymes, inhibited the two reactions to a comparable degree. The partial reversion by light of CO inhibition suggests the involvement of cytochrome P-450 in either reaction.

Table 1

Comparative effects of inhibitors on lauric acid hydroxylation and 9-DDNA epoxidation by tuber microsomes

Incubation conditions	Preincubation time (min)	% maximal activity	
		Lauric acid	9-DDNA
Complete	–	100[a] ± 1.6	100[b] ± 1.7
+ CO pur (30 s)	–	0	0
– NADPH	–	<2	<2
+ boiled microsomes	–	0	0
– NADPH + NADH (1 mM)	–	25 ± 1.5	15 ± 5.2
– NADPH + NADH (10 mM)	–	72 ± 0.6	56 ± 8.2
+ IgG (25 μl)	–	59 ± 2.9	47 ± 10.7
+ IgG (50 μl)	–	24 ± 1.3	21 ± 4.3
Complete with uninduced microsomes	–	5 ± 1.8	4 ± 2.3
Complete	5	100[c] ± 2.8	100[d] ± 1.8
+ 9-decenoate (50 μM)	5	70 ± 0.5	59 ± 2.7
+ 9-decenoate (50 μM)	10	31 ± 1.9	30 ± 6.5

Values are means ± SD (n = 4). Results are expressed in percent of maximal activity which was: [a] 271, [b] 201, [c] 153 and [d] 132 pmol/min per mg protein. Enzyme activities were assayed taking 8-, 9- and 10-hydroxylaurates as a bulk when lauric acid was the substrate and 9,10-epoxylaurate (*cis* + *trans*) plus 8-hydroxy-9-dodecenoate for 9-DDNA. Microsomes from uninduced tissues were prepared immediately after slicing Jerusalem artichoke tubers. Incubation conditions are described in section 2

Table 2

Partial reversion by light of CO inhibition of lauric acid hydroxylation and 9-DDNA 8-hydroxylation or 9,10-epoxidation

Substrates	Products	Incubation conditions		Reversion of CO inhibition (%)
		Air (control)	CO/air (% inhibition)	
Laurate	8-, 9-, 10-hydroxy-	100[a] ± 4.6	light 22.5 ± 7.2 dark 36.1 ± 4.6	37.6
9-DDNA	9,10-epoxy- (*cis*+*trans*)	100[b] ± 3.6	light 22.1 ± 3.7 dark 43.7 ± 4.9	51.7
	8-hydroxy-dodecenoate	100[c] ± 3.8	light 17.2 ± 3.7 dark 37.5 ± 4.9	51.4

Values are means ± SD (n = 4). Inhibition is expressed in % of the maximum rate of product formation which was: [a] 351, [b] 247 and [c] 30 pmol/min per mg protein. Incubation conditions are given in section 2

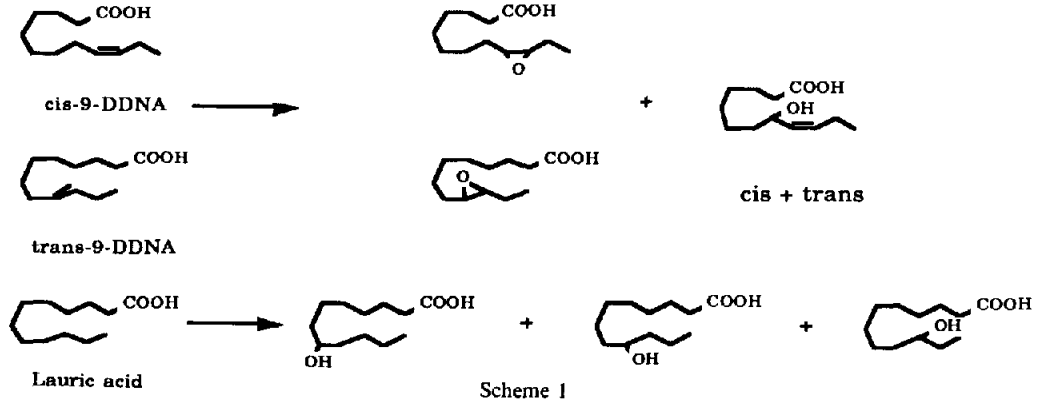
The calculated partition coefficients between light and dark incubations with lauric acid or 9-DDNA and CO [(laurate) K_{light} = 4.31, K_{dark} = 2.21; (9-DDNA) K_{light} = 4.85, K_{dark} = 1.71)] were similar. Finally, we found that 9-decenoic acid, a terminal olefin which is a selective and irreversible inactivator of lauric acid in-chain hydroxylase [20,21], produced a strong and comparable inhibition of epoxidation of 9-DDNA and hydroxylation of laurate in microsomes preincubated with NADPH and the suicide substrate.

4. DISCUSSION

The present work was undertaken in order to explore the mechanisms of enzymatic epoxidation of fatty acids in plants. Dodecenoic acids were chosen as model substrates because the hydroxylation of lauric acid by cytochrome P-450 monooxygenases is well documented and occurs in animals, plants and microorganisms. Almost all plants, apart from Leguminosae which ω-hydroxylate this fatty acid, catalyse in-chain hydroxylation of laurate, primarily at position 9. This position is also of strategic importance for the oxidation of most long-chain polyunsaturated fatty acids, since it is the site of attack for desaturases (yielding Δ9-enoates) and oxygenases to produce epoxides and hydroperoxides.

Jerusalem artichoke microsomes actively catalyzed the epoxidation of 9-DDNA. This reaction is not plant-specific, since microsomes from other in-chain hydroxylating species such as *Tulipa* produce the same pattern of reaction products (not shown). Naturally occurring unsaturated fatty acids are *cis* and, to our knowledge, no *trans*-epoxidase has yet been described. It is striking therefore that the *trans* isomer appeared to be as good a substrate as the *cis* form, as shown by the nearly identical rates of metabolism determined for both isomers. Furthermore, the reaction proceeds with total retention of configuration, since no detectable *trans*-epoxide was formed from *cis*-9-DDNA and vice versa. This stereochemical integrity implies that during oxidation of both substrates, no intermediate capable of rotation around the C9-C10 axis is formed.

It is likely that epoxidation of *cis*- and *trans*-9-DDNA is catalyzed by the same epoxidase, since the ratio of *cis*- to *trans*-epoxides formed did not change according to microsomal preparations, incubation conditions and use of several inhibitors like CO, suicide substrate or anti-reductase antibody. Strong supportive evidence for the cytochrome P-450 nature of the enzyme is provided by the microsomal localization of the epoxidase, its requirement for O_2 and NADPH, the inhibitory effects of antibody against NADPH-cytochrome P-450 reductase, of CO and of 9-decenoate, a mechanism-based inactivator of IC-LAH. The possibility that epoxidation and hydroxylation are catalyzed by the same or closely related cytochromes P-450 is suggested by identical sensitivity toward CO, electron donors, suicide substrate, anti-reductase, cross-inhibition of reactions by laurate or 9-DDNA and stimulation to the same extent of both activities in Jerusalem artichoke pretreated with aminopyrine and various inducers such as phenobarbital or manganese ions (not shown). The position but not the geometry of the double bond seems to be important for the epoxidation reaction as shown in scheme 1:



Scheme 1

In addition to the 9,10-epoxides, 8-hydroxy-9-dodecenoic acid is also formed from 9-DDNA. Thus, it appears that carbons C8, C9 and C10 are the selective targets of oxidative attack by plant cytochrome(s) P-450 in 9-DDNA as well as in lauric acid. The regioselectivity of the epoxygenase is now being further investigated using 7-, 8- and 10-DDNA as model substrates. Preliminary results confirm the high regioselectivity of the enzyme.

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