Production *in vitro* by the cytochrome P450 CYP94A1 of major C_{18} cutin monomers and potential messengers in plant–pathogen interactions: enantioselectivity studies

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The major C_{18} cutin monomers are 18-hydroxy-9,10-epoxystearic and 9,10,18-trihydroxystearic acids. These compounds are also known messengers in plant–pathogen interactions. We have previously shown that their common precursor 9,10-epoxystearic acid was formed by the epoxidation of oleic acid in *Vicia sativa* microsomes (Pinot, Salaün, Bosch, Lesot, Mioskowski and Durst (1992) Biochem. Biophys. Res. Commun. **184**, 183–193). Here we determine the chirality of the epoxide produced as (9*R*,10*S*) and (9*S*,10*R*) in the ratio 90:10 respectively. We further show that microsomes from yeast expressing the cytochrome P450 CYP94A1 are capable of hydroxylating the methyl terminus of 9,10-epoxystearic and 9,10-dihydroxystearic acids in the presence of NADPH to form the corresponding 18-hydroxy derivatives. The reactions were not catalysed by microsomes from yeast transformed with a void plasmid or in absence of NADPH. After incubation of a synthetic racemic mixture of 9,10-epoxystearic acid with microsomes of yeast expressing CYP94A1, the chirality of the residual epoxide was shifted to 66:34 in favour of the (9*S*,10*R*) enantiomer. Both enantiomers were incubated separately and V_{max}/K_m values of 16 and 3.42 ml/min per nmol of P450 for (9*R*,10*S*) and (9*S*,10*R*) respectively were determined, demonstrating that CYP94A1 is enantioselective for the (9*R*,10*S*) enantiomer, which is preferentially formed in *V. sativa* microsomes. Compared with the epoxide, the diol 9,10-dihydroxystearic acid was a much poorer substrate for the ω-hydroxylase, with a measured V_{max}/K_m of 0.33 ml/min per nmol of P450. Our results indicate that the activity of CYP94A1 is strongly influenced by the stereochemistry of the 9,10-epoxide and the nature of substituents on carbons 9 and 10, with V_{max}/K_m values for epoxide $\gg$ oleic acid $>$ diol.

Key words: chirality, epoxide, fatty acid, ω-hydroxylase.

INTRODUCTION

For a long time, ω-hydroxy fatty acids have been considered only as passive constituents of cutin, a component of the cuticle, the first barrier that protects plants against chemical or biological stresses [1]. Cutin is a biopolymer in which fatty acids, mainly from the C_{16} and C_{18} families, are cross-linked by ester bonds between carboxy and ω-hydroxy groups. In the C_{18} family, 9,10,18-trihydroxystearic and 18-hydroxy-9,10-epoxystearic acids are the major monomers [1]. More recently it has been demonstrated that besides being constituents of protective layers, ω-hydroxy fatty acids are fundamental in plant–pathogen interactions. Spores of virulent fungi carry cutinases, which hydrolyse cutin when they come into contact with plants [2]. The release of cutin monomers is a major event during the infection of plants by fungal pathogens. The monomers have been shown to enhance the transcription of cutinase genes [3–5]. They also stimulate the formation of an appressorium, a specialized infection structure that facilitates the penetration of the fungus [6,7]. However, the importance of cutinase during infection is controversial. Several studies based on the use of cutinase-lacking mutants demonstrate that cutinase is not essential for the pathogenicity of various fungi [8–10]. In contrast, bean leaves treated with purified cutinase become protected against infection by *Rhizoctonia solani* [11]. The protective mechanism remains unclear, but Schweizer et al. [12,13] clearly demonstrated with different models that pathogen-challenged plants perceived cutin monomers as endogenous molecules for the induction of resistance. Also, an enhanced elicitation of H_2O_2 was reported in cucumber hypocotyls in the presence of cutin monomers [14]. It is striking that 9,10,18-trihydroxystearic and 18-hydroxy-9,10-epoxystearic acids, which are the major C_{18} cutin monomers [1], also have the strongest effect in eliciting defence mechanisms and H_2O_2 production.

We have shown previously that when incubated with *Vicia sativa* microsomes, oleic acid was converted into 9,10-epoxystearic acid [15], which could be hydrolysed to the diol 9,10-dihydroxystearic acid by an epoxide hydrolase [16]. An enzymic system located in the microsomes could generate 9,10,18-trihydroxystearic and 18-hydroxy-9,10-epoxystearic acids by ω-hydroxylation of both the epoxide and the diol [15]. The subcellular location, the requirement for NADPH, inhibition studies with carbon monoxide and antibodies raised against NADPH:P450 reductase unequivocally demonstrated the involvement of a cytochrome P450-dependent ω-hydroxylase in these latter reactions. We recently cloned the cytochrome P450 CYP94A1 from *V. sativa* [17]. When expressed in yeast, CYP94A1 catalyses the ω-hydroxylation of saturated and unsaturated fatty acids. Treatment of *V. sativa* seedlings with the plant hormone methyl jasmonate leads to a rapid (1 h) accumulation of CYP94A1 transcripts, suggesting a possible involvement in plant defence [18].

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Here we demonstrate that CYP94A1 is capable of catalysing the formation of 18-hydroxy-9,10-epoxystearic and 9,10,18-trihydroxystearic acids by the ω-hydroxylation of 9,10-epoxystearic and 9,10-dihydroxystearic acids. We previously reported the presence of an epoxygenase in *V. sativa* microsomes. Here we examine the stereochemistry of oleic acid epoxidation catalysed by the epoxygenase and we demonstrate that CYP94A1 is enantioselective for the (9*R*,10*S*)-epoxystearic acid preferentially formed by the epoxygenase. This is the first report to our knowledge about the enantioselectivity of a fatty acid ω-hydroxylase. Our results further indicate that the affinity of CYP94A1 for related C_{18} fatty acids is dependent on the nature of substituents of C-9 and C-10 and on the stereochemistry of the 9,10-epoxide.

EXPERIMENTAL

Chemicals

[1-^{14}C]Lauric acid (45 Ci/mol) was purchased from CEA (Gif sur Yvette, France). [1-^{14}C]Oleic acid (50 Ci/mol) was from NEN-Dupont (Stevenage, Herts., U.K.). A racemic sample of (*Z*)-9,10-epoxy[1-^{14}C]stearic acid was synthesized from [1-^{14}C]oleic acid by using *m*-chloroperoxybenzoic acid. The corresponding diol, 9,10-dihydroxy[1-^{14}C]stearic acid, was prepared by acidic hydrolysis of the epoxide.

Silylating reagent, *N*,*O*-bistrimethylsilyltrifluoroacetamide containing 1% (v/v) trimethylchlorosilane, was from Pierce Europe (Ound-Beijerland, The Netherlands). NADPH was from Sigma Chimie (La Verpillière, France). Thin-layer plates (silica gel G60 F254, 0.25 mm) were from Merck (Darmstadt, Germany).

Preparation of *V. sativa* subcellular fractions

Etiolated seedlings 4 days old (approx. 30 g) grown at 26 °C on wet paper were harvested and homogenized with an Ultra-Turrax homogenizer (15000 rev./min, twice for 30 s each) at 4 °C in a final volume of 100 ml of 100 mM sodium phosphate buffer, pH 7.4, containing 250 mM sucrose, 40 mM sodium ascorbate, 10 mM 2-mercaptoethanol and 1 mM PMSF. The homogenate was filtered through 50 μm Blutex cloth and centrifuged for 10 min at 10000 ***g***. The resulting supernatant was centrifuged for 1 h at 100000 ***g***. The soluble fraction was divided into aliquots and stored at −30 °C. To eliminate contamination from the soluble fraction, the microsomal pellet was homogenized with a Potter–Elvehjem homogenizer in 100 mM pyrophosphate buffer, pH 7.5, containing 1.5 mM 2-mercaptoethanol. After a second centrifugation at 100000 ***g***, the microsomal pellet was resuspended in 7 ml of 100 mM sodium phosphate buffer, pH 7.4, containing 30% (v/v) glycerol, then divided into aliquots and stored at −30 °C. Protein concentrations of the microsomal and soluble fractions were estimated with a microassay from Bio-Rad, with BSA as a standard. The cytochrome P450 content was measured by the method of Omura and Sato [19].

Heterologous expression of CYP94A1 in yeast

CYP94A1 was expressed in *Saccharomyces cerevisiae* as described previously [17]. Yeast cells were harvested, broken with glass beads (0.45 mm diameter) and microsomes were prepared by differential centrifugation as described above for microsomes of *V. sativa* seedlings. Microsomal membranes were resuspended in 100 mM sodium phosphate buffer, pH 7.4, containing 30% (v/v) glycerol and 1.5 mM 2-mercaptoethanol.

Enzyme activities

All radiolabelled substrates were dissolved in ethanol, which was evaporated off before the addition of microsomes to the glass tube. Resolubilization of the substrates was confirmed by measuring the radioactivity of the incubation medium.

Epoxidation of radiolabelled oleic acid (100 μM) by *V. sativa* microsomes was performed in a final volume of 0.2 ml of 20 mM sodium phosphate buffer, pH 7.4. The reaction was initiated by the addition of 0.3 mg of microsomal protein and stopped after 5 min by the addition of 0.1 ml of acetonitrile (containing 0.2% acetic acid). The reaction products were resolved by TLC as described below.

CYP94A1 ω-hydroxylase activities were determined by following the formation rate of hydroxylated products. The standard assay (0.2 ml) contained 20 mM sodium phosphate buffer, pH 7.4, containing 1 mM NADPH, a regenerating system (consisting of a final concentration of 6.7 mM Glc-6-P and 0.4 unit of Glc-6-P dehydrogenase) and radiolabelled substrate (100 μM). The reaction was initiated by the addition of NADPH and was stopped by the addition of 0.1 ml of acetonitrile (containing 0.2% acetic acid). The reaction products were resolved by TLC as described below. Kinetic studies were performed for 7 min at 27 °C with 0.7 and 21 pmol of CYP94A1 for 9,10-epoxystearic and 9,10-dihydroxystearic acids respectively. The determination of K_m and V_{max} was based on six data points from 0.46 to 15 μM for 9,10-epoxystearic acid and from 1.75 to 100 μM for 9,10-dihydroxystearic acid.

Chromatographic methods

Incubation media were directly spotted on TLC plates, which were developed with a mixture of diethyl ether/light petroleum (boiling range 40–60 °C)/formic acid (50:50:1, by vol.). The plates were scanned with a thin-layer scanner (Berthold LB 2723). The area corresponding to the metabolites was scraped into counting vials and quantified by liquid scintillation, or the compounds were eluted from the silica with 10 ml of diethyl ether/hexane (50:50, v/v), which was removed by evaporation. They were then subjected to HPLC analysis.

Chiral analysis

Chiral analyses were performed as previously described by using optically pure synthetic (9*R*,10*S*)-epoxystearate methyl ester as a standard [16]. In brief, radiolabelled enantiomers of 9,10-epoxystearic acid were separated by HPLC (Waters, equipped with two 510 pumps and a U6K injector from Waters) with the use of a chiral column [Chiracel OB (4.6 mm × 250 mm); J. J. Baker Chemical Co.]. Enantiomers were resolved with an isocratic solvent: hexane/propan-2-ol/acetic acid (99.7:0.2:0.1, by vol.) at a flow rate of 0.8 ml/min. The same conditions were used to resolve enantiomers of 9,10-epoxystearate methyl ester. The radioactivity of the HPLC effluent was monitored with a computerized on-line solid-scintillation counter (Ramona-D; RAYTEST, Straubenhardt, Germany). Under the present conditions of analysis, the (9*S*,10*R*)- and (9*R*,10*S*)-epoxystearic acids have retention times of 61 and 68 min respectively and the corresponding methyl esters have retention times of 27 and 37 min respectively.

The chirality of oleic acid epoxidation in *V. sativa* microsomes was determined after separation on TLC of the product 9,10-epoxystearic from oleic acid as described above. The epoxide was eluted from the silica with 10 ml of diethyl ether/hexane (50:50, v/v), which was removed by evaporation. The epoxide was dissolved in hexane (40 μl) before analysis by HPLC.

To determine the chirality of the residual epoxide after incubation with CYP94A1, the epoxide and the ω-hydroxylated product were separated by TLC. The area corresponding to the epoxide was scraped and the epoxide was eluted from the silica. The residual epoxide was dissolved in hexane (40 μl) after methylation with diazomethane, then analysed by HPLC.

GLC/MS

Metabolites generated during the incubation of 9,10-epoxystearic and 9,10-dihydroxystearic acids with CYP94A1 were eluted from silica with 10 ml of diethyl ether/hexane (50:50, v/v), methylated with diazomethane and silylated with a mixture of pyridine and *N*,*O*-bistrimethylsilyltrifluoroacetamide containing 1% (v/v) trimethylchlorosilane (1:1, v/v). They were then subjected to GLC/MS analysis (electron-impact mode, 70 eV) as described previously [20]. The mass spectrum of the methyl ester trimethylsilyl ether derivative of the metabolite (Figure 1B, peak 2) generated from 9,10-epoxystearic acid showed ions at m/z 73 [$(CH_3)_3Si$], 103 [$(CH_3)_3SiO^+{=}CH_2$], 199, 243, 385 ($M-15$), 369 ($M-31$) and 353 ($M-47$). This fragmentation pattern is characteristic of the methyl ester trimethylsilyl ether derivative of 18-hydroxy-9,10-epoxystearic acid [21]. The mass spectrum of the methyl ester trimethylsilyl ether derivative of the metabolite (Figure 1D, peak 4) generated from 9,10-dihydroxystearic acid showed ions at m/z 73, 103, 259, 303 and 332, characteristic of the derivative of 9,10,18-trihydroxystearic acid [21].

RESULTS

ω-Hydroxylation of 9,10-epoxystearic and 9,10-dihydroxystearic acids

Throughout this study we used microsomes from yeast strain WAT11 transformed with CYP94A1 [17] as a source of functional enzyme. Yeast has only three cytochromes P450; they are expressed at a negligible rate under the growth conditions used [22]. None of these P450s is able to oxidize fatty acids. Furthermore, strain WAT11 overexpresses a plant P450 reductase that ensures excellent electron transfer and probably increases the stability of the plant P450 in the yeast endoplasmic reticulum. Microsomes from yeast transformed with the void vector served as control.

After incubation of (*Z*)-9,10-epoxy[1-^{14}C]stearic acid with microsomes and NADPH, the incubation media were spotted directly on TLC plates. Figure 1 shows radiochromatograms obtained after incubation in the absence (Figure 1A) or the presence (Figure 1B) of NADPH. No metabolites were formed in the absence of NADPH (Figure 1A). The addition of NADPH led to the formation of a polar metabolite (Figure 1B, peak 2). The compound was not produced after incubation with boiled microsomes or with microsomes from control yeast. After purification, this metabolite was identified as 18-hydroxy-9,10-epoxystearic acid by GLC/MS analysis.

Similarly, with 9,10-dihydroxy[1-^{14}C]stearic acid as substrate, CYP94A1 produced only one metabolite, which was identified by GLC/MS analysis as 9,10,18-trihydroxystearic acid (Figure 1D, peak 4). The compound was not formed with boiled microsomes, control microsomes or in the absence of NADPH (Figure 1C).

Stereochemistry of oleic acid epoxidation in *V. sativa* microsomes

We have previously shown that oleic acid is epoxidized when incubated with *V. sativa* microsomes [15]. We have now determined the absolute stereochemistry of the epoxide generated. Oleic acid was incubated with the microsomal fraction of *V. sativa*. The 9,10-epoxystearic acid produced was separated from oleic acid on TLC, eluted from silica and subjected to HPLC analysis. Results are presented in Figure 2. The epoxide was a mixture of (9*R*,10*S*) and (9*S*,10*R*) in the ratio 90:10 respectively,

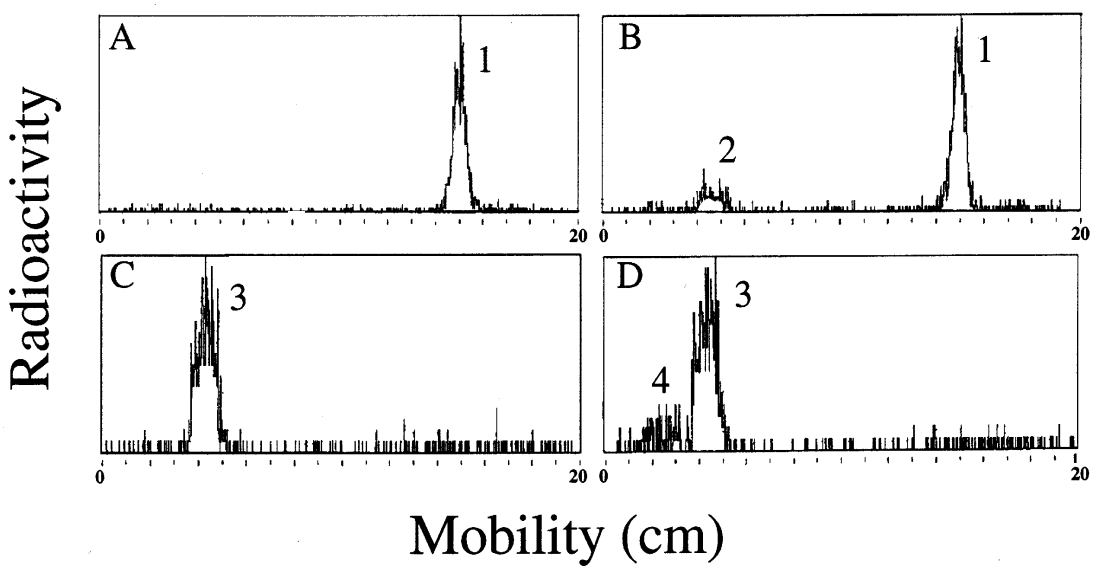

Figure 1 Radiochromatographic resolution by TLC of metabolites generated in incubations with microsomes of yeast expressing CYP94A1

Microsomes were incubated with 100 μM of 9,10-epoxy[1-^{14}C]stearic acid (**A**, **B**) or with 100 μM of 9,10-dihydroxy[1-^{14}C]stearic acid (**C**, **D**) in the absence (**A**, **C**) or the presence (**B**, **D**) of NADPH. Incubations were performed at 27 °C and contained 4.5 and 22.5 pmol of CYP94A1 for 9,10-epoxystearic and 9,10-dihydroxystearic acids respectively; they were stopped after 7 min by the addition of acetonitrile (containing 0.2% acetic acid) and spotted directly on TLC plates [diethyl ether/light petroleum (boiling range 40–60 °C)/formic acid (50:50:1, by vol.)]. Peak 1, 9,10-epoxystearic acid; peak 2, 18-hydroxy-9,10-epoxystearic acid; peak 3, 9,10-dihydroxystearic acid; peak 4, 9,10,18-trihydroxystearic acid.

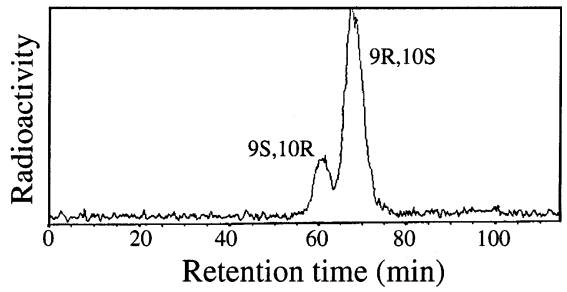

Figure 2 HPLC elution profile of the enantiomers of 9,10-epoxy[1-^{14}C]stearic acid formed during the incubation of [1-^{14}C]oleic acid with *V. sativa* microsomes

V. sativa microsomes (0.3 mg of protein) were incubated for 5 min at 27 °C with 100 μM of [1-^{14}C]oleic acid. After the reaction had been stopped with 0.1 ml of acetonitrile (containing 0.2% acetic acid), oleic acid and the corresponding epoxide were resolved by TLC. The epoxide was eluted from silica and subjected to HPLC analysis with hexane/propan-2-ol/acetic acid (99.7:0.2:0.1, by vol.) at a flow rate of 0.8 ml/min.

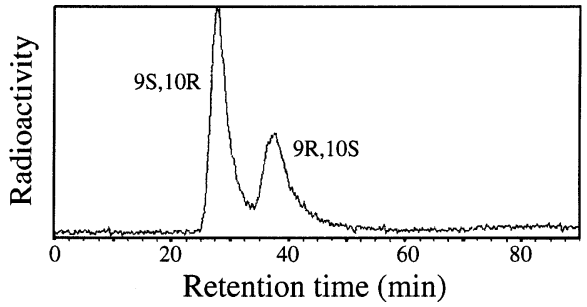

Figure 3 HPLC elution profile of the methyl ester of the residual enantiomers of 9,10-epoxy[1-^{14}C]stearic acid after incubation with microsomes of yeast expressing CYP94A1

Microsomes were incubated with 100 μM of racemic 9,10-epoxy[1-^{14}C]stearic acid. After 30% of the epoxide had been ω-hydroxylated, the remaining 70% was purified by TLC, then methylated and subjected to HPLC with hexane/propan-2-ol/acetic acid (99.7:0.2:0.1, by vol.) at a flow rate of 0.8 ml/min.

thus showing a large enantiomeric excess in favour of the (9*R*,10*S*) enantiomer.

Enantioselectivity of CYP94A1

Two different experiments were performed to determine the enantioselectivity of CYP94A1 towards the epoxide. In the first experiment, the racemic mixture of the epoxide was incubated with yeast microsomes and the reaction was stopped before more than 30% of the substrate had been hydroxylated. The residual epoxide was separated from its ω-hydroxy derivative by TLC, eluted from the silica, methylated with diazomethane and subjected to HPLC analysis. Figure 3 shows that the residual epoxide was no longer racemic and was a 66:34 mixture, with the (9*S*,10*R*) enantiomer in excess. Methylation of the carboxy group did not improve the separation but shortened the retention time of the enantiomers in comparison with the experiment shown in Figure 2. The second experiment studied whether this enantioselectivity resulted from differences in K_m or in V_{max}. The two enantiomers of 9,10-epoxystearic acid were separated and purified by HPLC (see the Experimental section). Six concentrations, ranging from 0.46 to 15 μM, of each enantiomer were

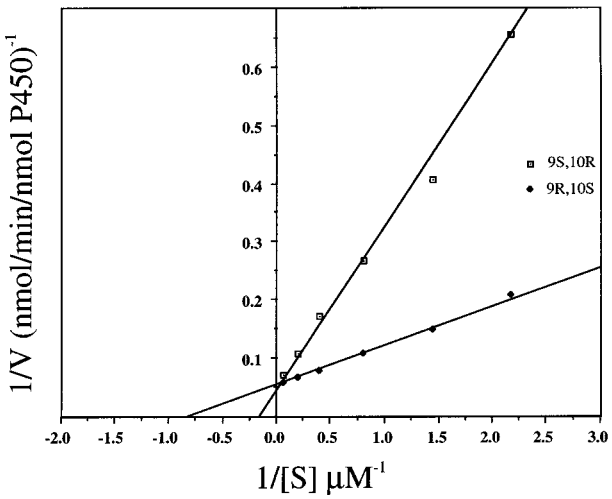

Figure 4 Lineweaver–Burk plot of ω-hydroxylation of enantiomers of 9,10-epoxy[1-^{14}C]stearic acid by CYP94A1

Microsomes of yeast (4.5 pmol of CYP94A1) were incubated for 7 min at 27 °C with purified enantiomers at concentrations ranging from 0.46 to 15 μM. Reactions were stopped with 0.1 ml of acetonitrile (containing 0.2% acetic acid); ω-hydroxylated products were then quantified after separation from the substrate by TLC. [S], substrate concentration. Results are means for duplicate experiments.

Table 1 Catalytic efficiency of CYP94A1 for the ω-hydroxylation of related C_{18} fatty acids

V_{max} and K_m for $C_{18:1}$ are from [17]. Abbreviation: n.d., not detectable.

Substrate	V_{max}/K_m (ml/min per nmol of P450)
$C_{18:0}$	n.d.
$C_{18:1}$	0.38
Diol	0.33
(9*S*,10*R*)	3.42
(9*R*,10*S*)	16.00

incubated separately with microsomes of yeast expressing CYP94A1 and the kinetics of ω-hydroxylation were determined. Figure 4 is a Lineweaver–Burk representation of the data. K_m and V_{max} for the (9*R*,10*S*) enantiomer were 1.2 ± 0.1 μM and 19.2 ± 0.3 nmol/min per nmol of P450 respectively. For the (9*S*,10*R*) enantiomer K_m and V_{max} values of 5.9 ± 0.1 μM and 20.2 ± 1.0 nmol/min per nmol of P450 were measured. The kinetic parameters clearly indicate that the enantioselectivity reflects a higher affinity of CYP94A1 for the (9*R*,10*S*) enantiomer rather than a better catalytic activity with this substrate.

Specificity of CYP94A1 for C_{18} substrates

The finding that CYP94A1 was enantioselective for the (9*R*,10*S*) epoxide prompted us to investigate the effect of the nature of the substituents and the stereochemistry of the 9,10-epoxide on the enzyme activity. Kinetic studies were performed with oleic acid, the two enantiomers of 9,10-epoxystearic acid and 9,10-dihydroxystearic acid. Microsomes of recombinant yeast were incubated with NADPH and different substrate concentrations. Table 1 shows the catalytic efficiency (V_{max}/K_m) of CYP94A1

with five related C_{18} fatty acids. The corresponding K_m values were 1.2 and 5.9 for the (9*R*,10*S*) and (9*S*,10*R*) epoxides, 26 μM for the 9,10-diol and 38 μM for oleic acid. Stearic acid ($C_{18:0}$) was neither metabolized nor recognized by the enzyme. This clearly shows a structure–activity relation based on the nature of the substitution on C-9 and C-10.

DISCUSSION

Hydroxylation of the methyl-terminus of 9,10-epoxystearic and 9,10-dihydroxystearic acids is a key reaction in the context of plant resistance because the ω-hydroxy derivatives of these compounds are the major cutin monomers of the C_{18} family [1]. Furthermore, there is increasing evidence of their involvement in plant–pathogen interactions. A better understanding of their role should be gained through the identification and characterization of the enzyme that is implicated in their formation.

We previously reported the presence in *V. sativa* microsomes of a cytochrome P450-dependent ω-hydroxylase that oxidizes 9,10-epoxystearic acid and the corresponding diol [15]. The enzymic studies of these reactions were complicated by the presence in this subcellular fraction of an epoxygenase and an epoxide hydrolase. The epoxidation reaction of oleic acid [15] did not require NADPH, indicating that cytochrome P450 was not involved. The enzyme catalysing this reaction was probably similar to that described by Hamberg and Hamberg [23] in microsomes of *Vicia faba*. They demonstrated that the epoxygenase was able to produce 9,10-epoxystearic acid by using hydroperoxide generated from endogenous fatty acids present in the microsomes as oxygen donor. Both enzymes from *V. sativa* and *V. faba* are microsomal and the epoxide formed presented an enantiomeric excess in favour of (9*R*,10*S*), reflecting similar steric constraints in the active site.

Recently we cloned CYP94A1 from *V. sativa* [17]. When expressed in *S. cerevisiae*, CYP94A1 catalyses the ω-hydroxylation of saturated and unsaturated fatty acids; the highest efficiency is towards lauric acid. This expression system has the advantage that there is no identifiable epoxide hydrolase homologue in the genome of *S. cerevisiae* and, indeed, biochemical studies in our laboratory failed to detect any epoxygenase or epoxide hydrolase activity. With the use of this system we demonstrate here that CYP94A1 is also able to hydroxylate 9,10-epoxystearic acid and the corresponding diol. As already observed for the oxidation of saturated or unsaturated fatty acid substrates of different chain lengths [17], CYP94A1 exclusively catalyses the oxidation of the methyl-terminus of the two oxygenated C_{18} fatty acids. This further confirms the high regioselectivity of CYP94A1.

With *V. sativa* microsomes we showed that mainly (9*R*,10*S*)-epoxystearic acid was produced from oleic acid. We therefore investigated whether CYP94A1 could discriminate between the two enantiomers. Determination of the chirality of the residual epoxide after the incubation of a racemic mixture with CYP94A1 showed that the enzyme did indeed ω-hydroxylate the (9*R*,10*S*) form preferentially. The apparent K_m for (9*R*,10*S*) was one-fifth that for (9*S*,10*R*). The enantioselectivity for the (9*R*,10*S*) form preferentially produced in microsomes, together with a K_m in the micromolar range, strongly suggest that (9*R*,10*S*)-epoxystearic acid is a physiological substrate of CYP94A1. This oxygenated fatty acid is metabolized with a catalytic efficiency (V_{max}/K_m) 10-fold that determined for lauric acid, the best substrate previously described [17].

The regioselectivity and enantioselectivity of CYP94A1 reflect steric constraints on the substrate in the active site. Our results show that the apparent affinity of CYP94A1 for the C_{18} fatty acids is highly substituent-dependent at positions 9 and 10. Because the affinities measured are not related to the solubility of the fatty acids in the incubation medium, the differences in kinetic parameters cannot be explained by differing physical properties. Stearic acid was not metabolized by CYP94A1; oleic acid, which differs by a double bond between C-9 and C-10, was metabolized with a K_m of 38 μM. The introduction of an epoxide function at this position decreased the K_m to 1.2 and 5.9 μM, depending on its chirality. This demonstrates that the presence of a polar character at that position increases the affinity. The higher K_m determined for the diol (26 μM) might be due to steric hindrance of the vicinal hydroxy groups. It is interesting to note that the plant fatty acid desaturase Fad6s, a microsomal di-iron-dependent oxygenase, is able to recognize hydroxy or epoxy groups at C-9 to introduce regiospecifically a double bond in its substrate [24,25].

In animals, enzymes capable of hydroxylating the methyl-terminus of fatty acids have been extensively studied and classified in the CYP4 family [26]. In the yeast *Candida*, fatty acids and alkanes are ω-hydroxylated by the P450s from the CYP52 family [27]. Despite the tremendous quantity of data collected, the mechanism that defines the high regioselectivity of these enzymes has not been completely elucidated. Mutants of CYP4 family members have been constructed and their catalytic properties analysed [28,29]: Glu-320 and Asp-323 in CYP4A1 and Leu-131 in CYP4A11 determined the regiospecificity of the ω-hydroxylases. The mutants were constructed on the basis of alignments with $P450_{BM-3}$. This soluble bacterial P450 hydroxylates fatty acids at the ω-1, ω-2 and ω-3 positions [30]. Crystallographic studies showed that both extremities of fatty acids interact with amino acid residues, which were identified [31]. The carboxy group interacts with Tyr-51 and Arg-47; the methyl-terminus is sterically hindered by Phe-87, which corresponds to Leu-131 in CYP4A11. These interactions are responsible for the enzyme's regioselectivity. Plant P450s have been classified into two phylogenetic groups: the A group contains most plant P450s, which perform reactions typical of plants; the non-A group P450s catalyse the oxidation of sterols and fatty acids, reactions that are common to the different kingdoms of life [32]. Phylogenetic studies (F. Durst, unpublished work) indicate that CYP94A1 and CYP86A1, another plant fatty acid ω-hydroxylase, cluster away from the plant A group P450s and close to the bacterial, fungal and animal fatty acid hydroxylases of families CYP102, CYP52 and CYP4 respectively. Sequence alignments with these hydroxylases are now being used to direct mutation experiments of CYP94A1 at putative substrate-recognition sites.

In summary, we have shown that CYP94A1 is able to generate 18-hydroxy-9,10-epoxystearic and 9,10,18-trihydroxystearic acids *in vitro*. These two compounds are the major C_{18} cutin monomers and are described as potential messengers in different plant–pathogen interactions. We are in the process of growing tobacco lines (sense and anti-sense) with coding sequences of homologues of CYP94A1. The study of these transgenic tobacco plants should help us to assess the involvement of CYP94A1 in plant resistance to stress. We also report here, for the first time to our knowledge, the enantioselectivity of a fatty acid ω-hydroxylase. We are planning to use site-directed mutagenesis and suicide inhibitors [33,34] to identify amino acids at the active site to obtain insight into the mechanism of regioselectivity and enantioselectivity of CYP94A1.

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