ω-Hydroxylation of Oleic Acid in *Vicia sativa* Microsomes

**Inhibition by Substrate Analogs and Inactivation by Terminal Acetylenes**

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Oleic acid (18:1) is hydroxylated exclusively on the terminal methyl by a microsomal cytochrome P-450-dependent system (ω-OAH) from clofibrate-induced *Vicia sativa* L. (var minor) seedlings (F. Pinot, J.-P. Salaun, H. Bosch, A. Lesot, C. Mioskowski, F. Durst [1992] Biochem Biophys Res Commun 184: 183-193). This reaction was inactivated by two terminal acetylenes; (Z)-9-octadecen-17-ynoic acid (17-ODCYA) and the corresponding epoxide, (Z)-9,10-epoxystearic acid (17-EODCYA). Inactivation was mechanism-based, with an apparent binding constant of 21 and 32 µM and half-lives of 16 and 19 min for 17-ODCYA and 17-EODCYA, respectively. We have investigated the participation of one or more ω-hydroxylase isoforms in the oxidation of fatty acids in this plant system. Lauric acid (12:0) is ω-hydroxylated by the cytochrome P-450-ω-hydroxylase ω-LAH (J.-P. Salaun, A. Simon, F. Durst [1986] Lipids 21: 776-779). Half-lives of ω-OAH and ω-LAH in the presence of 40 µM 17-ODCYA were 23 and 41 min, respectively. Inhibition of oleic acid ω-hydroxylation was competitive with linoleic acid (18:2), but non-competitive with lauric acid (12:0). In contrast, oleic acid did not inhibit ω-hydroxylation of lauric acid. Furthermore, 1-pentadecyltriazole inhibited ω-hydroxylation of oleic acid but not of lauric acid. These results suggest that distinct monooxygenases catalyze ω-hydroxylation of medium- and long-chain fatty acids in *V. sativa* microsomes.

Oleic acid and its oxygenated derivatives form an important part of the fatty acids found in cuticular membranes of plants (Kolattukudy, 1981). Previous investigations from our laboratory have demonstrated that, in *Vicia sativa* L. (var minor) microsomes, oleic acid is subjected to a cascade of reactions that involve at least three distinct enzymes: a peroxidase, an epoxide hydrolase, and a Cyt P-450-dependent ω-hydroxylase (Pinot et al., 1992). The latter enzymic system is able to ω-hydroxylate oleic acid, (Z)-9,10-epoxystearic acid and 9,10-dihydroxystearic acid. The interplay of these enzymes may account for the formation of the major C_{18} cutin monomers derived from oleic acid (Kolattukudy, 1981).

Different studies performed with mammalian systems suggest that ω-hydroxylation by Cyt P-450 enzymes is the first step in fatty acid catabolism (Gibson, 1989). The inactivation of these ω-hydroxylases by terminal acetylenic compounds has been extensively studied (Kunze et al., 1983; Ortiz De Montellano and Reich, 1984). Acetylenic fatty acid analogs inactivate lauric acid ω-hydroxylases from rat liver both in vitro and in vivo (Cajacob and Ortiz De Montellano, 1986). Cyt P-450LAω, purified from clofibrate-induced rat liver, oxidizes lauric acid to 11- and 12-hydroxysterodecanoic acids in a 1:17 ratio (Cajacob et al., 1988). Inhibition of the enzyme with 10-undecyenoic acid leads to the inactivation of only one-half of the enzymic activity, suggesting that the preparation contained two distinct lauric acid ω-hydroxylases. Similarly, oxygenases, which ω-hydroxylate leukotrienes in human polymorphonuclear leukocytes (Shak et al., 1985) and prostaglandins in rabbit lung (Muerhoff et al., 1989), are inactivated by terminal acetylenic fatty acids. Shak et al. (1985) showed that 12-hydroxy-16-heptadecenoic acid inactivates rabbit lung microsomal prostaglandin ω-hydroxylase without affecting lauric acid ω-hydroxylase.

Few studies have addressed the autocatalytic inactivation of fatty acid ω-hydroxylases in plant systems. Earlier studies from our laboratory have shown that lauric acid (Salaun et al., 1986) and a series of C_{18} Z and E monounsaturated analogs (Weissbart et al., 1992) are ω-hydroxylated in *V. sativa* microsomes. These ω-hydroxylation reactions are in-

**Abbreviations:** 17-EODCYA, (Z)-9,10-epoxystearic acid, 17-ODCYA, (Z)-9-undecenoic acid; ω-LAH, omega hydroxylase of lauric acid; ω-OAH, omega hydroxylase of oleic acid.

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1 F.P. and H.B. are currently supported by fellowships from Ministère de la Recherche et de la Technologie and from Centre National de la Recherche Scientifique-Région Alsace. C.A. was supported by Rhône-Poulenc with the contribution of the Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace. C.A. was supported by fellowships from Ministère de la Recherche Scientifique-Région Alsace.

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hibited by 11-dodecynoic acid, the terminal acetylenic analog of lauric acid (Salaun et al., 1988; Weissbart et al., 1992).

Based on the results obtained with mammalian and plant systems, we have now synthesized (Z)-9-octadecen-17-ynoic acid and the corresponding (Z)-9,10-epoxide in an effort to inactivate the ω-hydroxylases involved in the oxidation of the C18 fatty acid family. These compounds are potential tools for investigating the mechanism of selective oxygenase inhibitors on the composition of the cuticle.

We report here, for the first time, the inactivation of an oleic acid ω-hydroxylase by acetylenic analogs of C18 fatty acids. To investigate the involvement of a single or of multiple forms of Cyt P-450 in ω-hydroxylation of lauric and oleic acids, competition and mechanism-based inactivation experiments were performed to compare the inhibition and inactivation of both activities.

MATERIALS AND METHODS

Chemicals

Radiolabeled [1,14C]-oleic acid (2.09 GBq/mmol) and [1,14C]-lauric acid (1.74 GBq/mmol) were from CEA (Gif sur Yvette, France). TLC plates (Silica gel G60 F254) were from Merck (Darmstadt, Germany). NADPH was purchased from Sigma Chimie (La Verpillière, France). 1-Pentadecyltriazole was a generous gift of Drs. Anding and Greiner from Rhône-Poulec Agrochimie (Lyon, France).

Synthesis of 17-ODCYA

8-Bromo-1-octanol was refluxed with 1.2 eq of triphenylphosphine, in anhydrous acetonitrile, overnight to give (7-hydroxyoctyl)-triphenylphosphonium bromide (yield: 95%). The alkylidene phosphorane of (7-hydroxyoctyl)-triphenylphosphonium bromide, generated by means of 2 eq of lithium diisopropylamide in tetrahydrofuran at -40°C, was reacted with 1 eq of 7-bromoheptanal to give 1-bromo-7-pentadecene-15-ol (yield: 66%). The alcohol was protected (Bollit and Mioskowski, 1988) as its tetrahydropyranyl ether (90%), with 86% yield and as a mixture Z/E of 79/21.

The alkylidenephosphorane of (7-hydroxyoctyl)-triphenylphosphonium bromide, generated by means of 2 eq of lithium diisopropylamide in tetrahydrofuran at -40°C, was reacted with 1.5 eq of the carbanion derived from trimethylsilylacetylene (generated from trimethylsilylacetone (97%). The resulting 1-trimethylsilyl-17-bromo-9-heptadecene-1-yne with 74% yield. The reagent PPh3/CBr4 then allows the conversion from the tetrahydropyranyl ether into the corresponding 1-trimethylsilyl-17-bromo-9-heptadecene-1-yne (55%). The resulting bromide is substituted by cyanide (as KCN) in DMSO at 80°C to 1-trimethylsilyl-17-cyano-9-heptadecene-1-yne (86%), which is finally hydrolyzed by 50% aqueous KOH/ethanol to 9-octadecene-17-ynoic acid with 86% yield and as a mixture Z/E of 79/21.

All intermediates were characterized by 1H and 13C NMR spectroscopy, IR spectroscopy, and elemental analysis.

Synthesis of 17-EODCYA

The experimental procedure is very close to phase transfer epoxidation by hydrogen peroxide described by Venturello et al. (1983). The epoxidation was performed on a 0.5-mmol 17-ODCYA scale, the phase transfer catalyst was Adogen 464, and the reaction time was 2 h. Yield was up to 79%.

Microsomal Preparations

Vicia sativa L. (var minor) seedlings were purchased from S.A. Blondeau (Bersie, France). Four-day-old etiolated seedlings were aged for 48 h in 1 mM clofibrate solution before isolation of the microsomal fractions as already described (Salaun et al., 1986). Microsomal proteins were quantified by a microassay procedure from Bio-Rad using BSA as a standard.

Enzyme Activities and Their Inhibitions

Enzyme activities were measured as described (Pinot et al., 1992; Weissbart et al., 1992) by following by TLC the rate of metabolite formation from radiolabeled substrates during incubation of microsomes from clofibrate-induced V. sativa seedlings. The metabolite generated by microsomes from oleic acid has been previously assigned to ω-hydroxyoleic acid (Pinot et al., 1992). Lauric acid was converted to 12-hydroxylaurate exclusively (Salaun et al., 1986). The standard assay contained 0.19 to 0.43 mg of microsomal protein, 20 mM phosphate buffer (pH 7.4), and radiolabeled substrate in a final volume of 0.2 mL. ω-Hydroxylase activities were measured in the presence of 1 mM NADPH plus a regenerating system (consisting of a final concentration of 6.7 mM Glc-6-P and 0.4 IU of Glc-6-P dehydrogenase) and 2.5 mM β-mercaptoethanol. Concentrations of substrates and inhibitors and times of incubation are mentioned in the legend of each figure. Solvents containing substrates or inhibitors were evaporated under a stream of argon before incubations. Reactions were initiated by adding NADPH at 27°C and stopped with 0.2 mL of acetonitril:acetic acid (99:0.8:2, v/v). Reaction products were extracted twice into 1 mL of diethyl ether and resolved by TLC.

Inactivation with Mechanism-Based Inhibitors

Following a procedure similar to that already described (Salaun et al., 1988), microsomes were preincubated at 27°C with 1 mM NADPH plus a regenerating system (see above) in the presence of different concentrations of (Z)-9-octadecen-17-ynoic or (Z)-9,10-epoxyoctadecan-17-ynoic acids. After different periods of time, a small volume (30 μL) of preincubated microsomes was added to incubation medium at 27°C that contained [1,14C]oleic acid or [1,14C]lauric acid, 1 mM NADPH, and phosphate buffer in a final volume of 0.2 mL. Incubations were allowed to continue a further 5 min at 27°C and then stopped as described above.

Chromatographic Analysis

Metabolites generated from incubations were resolved by silica gel TLC. Plates were developed with a mixture of diethyl ether:light petroleum (bp 40–60°C):formic acid (50:50:1, v/v/v). The areas corresponding to 18-hydroxylauric (Rf = 0.2) and 12-hydroxylauric (Rf = 0.2) acids were scraped directly into vials and quantified by liquid scintillation (Pinot et al., 1992).
RESULTS

Inactivation of ω-OAH by 17-ODCYA and 17-EODCYA

Microsomes from V. sativa seedlings were preincubated for various lengths of time with 1 mM NADPH and 17-ODCYA at concentrations ranging from 20 to 80 μM, and the residual ω-OAH activity was measured (Fig. 1). No inhibition was observed in incubations with 17-ODCYA alone. However, there was a slight but sizable loss of activity in the presence of NADPH alone. This was also observed in similar experiments with mammalian (Cajacob and Ortiz De Montellano, 1986) and other plant systems (Salaun et al., 1988). Preincubation with 17-ODCYA produced a time- and concentration-dependent inhibition of oleic acid hydroxylation. The inactivation rates followed roughly pseudo first-order kinetics, suggesting autocatalytic destruction of the enzyme. After correction for the activity loss observed with NADPH alone, a $K_i$ of 21 μM and a $k_{\text{kinactivation}}$ of 7.2 × 10^{-4} s^{-1} were calculated. The half-life of the enzyme at saturating inhibitor concentration was 16 min.

Recently, we demonstrated that cis-9,10-epoxystearic acid was ω-hydroxylated by a Cyt P-450 system in microsomes from V. sativa (Pinot et al., 1992). We have now synthesized 17-EODCYA, the terminal acetylene analog of this substrate, to determine whether ω-OAH might be implicated in this reaction. Preincubation of microsomes with 17-EODCYA produced a concentration- and time-dependent inactivation of ω-OAH similar to that produced by 17-ODCYA (data not shown). After correction for the effect of preincubation with NADPH alone, a $K_i$ of 32 μM and a $k_{\text{kinactivation}}$ of 6 × 10^{-4} s^{-1} were determined. The half-life of the enzyme at saturating 17-EODCYA concentration (19 min) was comparable to that determined for 17-ODCYA, suggesting that ω-OAH catalyzes the ω-hydroxylation of cis-9,10-epoxystearic acid.

Competition with Linoleic Acid

In addition to oleic acid derivatives, cutin contains minor amounts of oxidized derivatives of linoleic acid, the 18:2 analog of oleic acid. As shown in Figure 2, linoleic acid is a competitive inhibitor of ω-OAH. A $K_i$ of 200 μM was calculated, suggesting that the apparent affinity of ω-OAH for linoleic acid is considerably lower than the affinity for oleic acid ($K_m = 110$ μM).

Oleic and Lauric Acid Are Hydroxylated by Distinct Cyt P-450 Enzymes

We had previously reported that lauric acid is ω-hydroxylated by a Cyt P-450 enzyme (ω-LAH) in V. sativa microsomes (Salaun et al., 1986). ω-LAH was shown to be specific for short (C10) and medium (C12-C14) fatty acids (Simon, 1987). Microsomes were preincubated for different lengths of time with NADPH and 40 μM 17-ODCYA, and the residual ω-hydroxylase activities for oleic and lauric acids were measured (Fig. 3). Both activities were inactivated, but at different rates: the half-life for ω-LAH was 41 min compared with 23 min for ω-OAH. Without preincubation, 17-ODCYA had no competitive effect on ω-LAH activity. The different inactivation rates may indicate that ω-hydroxylation of oleic and lauric acids are catalyzed by distinct Cyt P-450 isoforms. To further investigate this hypothesis, we performed competition experiments. Figure 4 shows that lauric acid is a noncompetitive inhibitor of ω-OAH activity. In contrast, ω-LAH activity measured at 30 μM lauric acid was not affected by oleic acid at concentrations ranging from 125 to 375 μM (Fig. 5). Another type of Cyt P-450 inhibitor is provided by nitrogen-containing molecules, notably azoles, which inhibit Cyt P-450 by bonding to the heme iron (Ortiz De Montellano and Reich, 1986). We have studied the effect of 1-pentade-
Figure 3. Time-dependent inactivation of ω-OAH and ω-LAH activities by 17-ODCYA. ω-OAH (□) and ω-LAH (×) activities are plotted in a semilogarithmic fashion against time of microsome preincubation in the presence of NADPH plus 40 μM 17-ODCYA. Control activities were 76 and 407 pmol min⁻¹ mg⁻¹ protein for ω-OAH and ω-LAH, respectively. The concentration of oleic and lauric acid was 100 μM.

cyltriazole, an alkane azole analog, on the ω-hydroxylation of lauric and oleic acids (Fig. 6). Formation of 12-hydroxy-lauric acid was decreased by less than 10%, but that of 18-hydroxyoleic acid was reduced by 34% in the presence of 60 μM of the triazole.

DISCUSSION

The physiological roles of fatty acid ω-hydroxylases, in animals as well as in plants, remain obscure. In mammals, it has been demonstrated that 20-hydroxyeicosatetraenoic acid constricts the rat aortic ring (Schwarzman et al., 1989) and inhibits ion transport in the rabbit kidney loop of Henle (Escalante et al., 1991). The existence of isoenzymes that catalyze only this particular reaction, the specific induction of one of these isoenzymes by clofibrate, and the enhanced excretion of dicarboxylic acids under conditions of high fatty acid flux suggest that fatty acid ω-hydroxylation could be involved in a catabolic process in mammals (Tamburini et al., 1984). Interestingly, clofibrate also induces fatty acid oxidation by plants (Salau et al., 1986; Pinot et al., 1992). Furthermore, Palma et al. (1991) have shown that this hypolipidemic drug leads, as in mammalian systems, to proliferation of peroxisomes and mitochondria in leaves of Pisum sativum. These analogies suggest that ω-hydroxylation of fatty acids in plants could be the first step in their catabolism through β-oxidation. However, several lines of evidence also suggest that ω-hydroxylation of long-chain fatty acids may be implicated in biosynthetic pathways. Studies of the biosynthesis of plant cutins and suberins have shown that C₁₈ fatty acids are incorporated into the cuticle after ω-hydroxylation by Cyt P-450 isoenzymes (Kolattukudy, 1980). We have recently demonstrated that the ω-hydroxylation of oleic acid, 9,10-epoxysearic acid, and 9,10-dihydroxysearic acid is performed by a Cyt P-450 from V. sativa microsomes (Pinot et al., 1992). We hypothesize that this reaction controls the elongation of the cutin polymer and is therefore a key step in the synthesis of the plant cuticle. To test this hypothesis, we are now developing inhibitors of the C₁₈ ω-hydroxylase.

The two acetylenes 17-ODCYA and 17-EODCYA proved...
was 100
activities. Relative w-LAH (black bars) and w-OAH (white bars)
relative w-LAH (black bars) and w-OAH (white bars)
activities against 1-pentadecyltriazole concentration. Microsomal incubation time was 10 min. Substrate concentration
value is the mean
rate constants of 7.2
absence of substrate, may react with the enzyme and inacti-
prime factor in mechanism-based enzyme inactivation, but
slow rate, the medium-chain fatty acid hydroxylase w-LAH.
It is well established that affinity of the inhibitor is not the prime factor in mechanism-based enzyme inactivation, but
rather it is the ratio of alklylation over normal product formation that is important (Walsh, 1982). Because the different Cyt P-450 isoforms share basically the same reaction mechanism, even modest affinity of nontarget Cyt P-450 forms for an inactivator may produce their inactivation. Although oleic acid itself is not an inhibitor of w-LAH (Fig. 5), one could assume that the rigid and linear acetylene group of 17-
ODCYA modifies the conformation of the substrate and enables recognition by the enzyme active site.
One alternative explanation to our data would be that a fraction of lauric acid hydroxylation is catalyzed by w-OAH and is therefore sensitive to 17-ODCYA. In this context, the noncompetitive inhibition of w-OAH by laurate would be due to the binding of laurate at a different site of the heme pocket than oleate. To test further for the involvement of distinct Cyt P-450 forms in the w-hydroxylation of medium- and long-chain fatty acids, we have used an azole alkane analog 1-pentadecyltriazole. Azole compounds inhibit Cyt P-450 by forming a noncovalent bond with the iron of the prosthetic group (Ortiz De Montellano and Reich, 1986). The inhibitory character of the azole is here enhanced by the presence of an aliphatic chain that interacts with the hydrophobic domain of the active site. The inhibition by w-OAH was 4 times higher than that by w-LAH. This confirms earlier studies from our laboratory that showed that w-LAH is inhibited only by alkyltriazoles with aliphatic chains shorter than 13 carbon atoms (our unpublished data).
Our data are best reconciled with the existence of at least two distinct Cyt P-450-dependent w-hydroxylase systems in V. sativa microsomes: an w-LAH catalyzing the oxidation of lauric acid and different laurate analogs (Salaun et al., 1986; Simon, 1987; Weissbart et al., 1992), and an w-OAH that hydroxylates C18 fatty acids. This latter enzyme may also catalyze a part of lauric acid oxidation, as evidenced by the effects of 17-ODCYA and 1-pentadecyltriazole and the non-competitive inhibition of w-OAH by laurate. We have recently shown that linoleic acid is oxidized by Cyt P-450 in V. sativa microsomes (Pinot, 1992). The competitive inhibition of w-OAH by linoleate (Fig. 2) suggests that these two C18 fatty acids are substrates of the same Cyt P-450 enzyme.
The irreversibility of mechanism-based inactivators such as 17-ODCYA and 17-EODCYA makes them attractive as probes for the study of the biochemical and physiological roles of w-OAH. It remains to be shown that these compounds are active in vivo. In mammals, terminal acetylenic fatty acid analogs are rapidly inactivated in vivo by β-oxidation (Cajacob et al., 1988). To increase the efficacy of our inhibitors, we plan to replace the carboxylic acid group by a sulfate, which is not subjected to β-oxidation (Cajacob et al., 1988). Preliminary studies in our laboratory have shown that a carboxylic group can be replaced by a sulfate without affecting recognition of the molecule by plant w-hydroxylases. We have also synthesized radiolabeled [1-14C]17-ODCYA and [1-14C]17-EODCYA. These compounds provide new tools to study further the mechanism of enzyme inactivation. It has been suggested (Cajacob et al., 1988) that acetylenes inactivate w-hydroxylases by alkylating the Cyt P-450 apoprotein. These radiolabeled inactivators will now be used to test this hypothesis and may serve as markers during hydroxylase purification.

Received February 9, 1993; accepted May 7, 1993.
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Figure 6. Effect of 1-pentadecyltriazole on w-OAH and w-LAH activities. Relative w-LAH (black bars) and w-OAH (white bars) activities are plotted against 1-pentadecyltriazole concentration. Microsomal incubation time was 10 min. Substrate concentration was 100 μM. Results are expressed as percent of maximal activities measured without 1-pentadecyltriazole, which were 99 and 323 pmol min−1 mg−1 protein for w-OAH and w-LAH, respectively. Each value is the mean of duplicate experiments.

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