Plant Physiol. (1992) 100, 874-881
0032-0889/92/100/0874/08/$01.00/0

Received for publication March 3, 1992
Accepted June 2, 1992

Aryl Hydroxylation of the Herbicide Diclofop by a Wheat Cytochrome P-450 Monooxygenase[1]

Substrate Specificity and Physiological Activity

Alfred Zimmerlin[2]* and Francis Durst

Département d'Enzymologie Cellulaire et Moléculaire, Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique UPR 406, 28, rue Goethe, F-67083 Strasbourg Cedex, France

ABSTRACT

Wheat (*Triticum aestivum* L. cv Etoile de Choisy) microsomes catalyzed the cytochrome P-450-dependent oxidation of the herbicide diclofop to three hydroxy-diclofop isomers. Hydroxylation was predominant at carbon 4, with migration of chlorine to carbon 5 (67%) and carbon 3 (25%). The 2,4-dichloro-5-hydroxy isomer was identified as a minor reaction product (8%). Substrate-specificity studies showed that the activity was not inhibited or was weakly inhibited by a range of xenobiotic or physiological cytochrome P-450 substrates, with the exception of lauric acid. Wheat microsomes also catalyze the metabolism of the herbicides chlorsulfuron, chlortoluron, and 2,4-dichlorophenoxyacetic acid and of the model substrate ethoxycoumarin, as well as the hydroxylation of the endogenous substrates cinnamic and lauric acids. Treatments of wheat seedlings with phenobarbital or the safener naphthalic acid anhydride enhanced the cytochrome P-450 content of the microsomes and all related activities except that of cinnamic acid 4-hydroxylase, which was reduced. The stimulation patterns of diclofop aryl hydroxylase and lauric acid hydroxylase were similar, in contrast with the other activities tested. Lauric acid inhibited competitively ($K_i = 9$ μM) the oxidation of diclofop and reciprocally. The similarity of diclofop aryl hydroxylase and lauric acid hydroxylase was further investigated by alternative substrate kinetics, autocatalytic inactivation, and computer-aided molecular modelisation studies, and the results suggest that both reactions are catalyzed by the same cytochrome P-450 isozyme.

The pharmacological and physiological significance of the Cyt P-450-mediated metabolism of foreign compounds in mammals has attracted considerable interest for many years (17). By comparison, the metabolism of xenobiotics by Cyt P-450 in plants is much less documented, because few laboratories have been successful in isolating active Cyt P-450 from plants (3). However, there is mounting evidence that Cyt P-450 reactions are responsible for the detoxification of certain herbicides in wheat (12, 15, 25), maize (4, 5, 13), and other crop species (14) and that these reactions can enable certain crop species to tolerate a herbicide.

It is well established that mammalian cytochrome P-450 systems exhibiting broad and overlapping substrate specificity may be rapidly induced by exposure to various xenobiotics, which leads to increased capacity to detoxify these compounds (6). It is believed that these Cyt P-450 forms evolved under the pressure of the wide range of toxic compounds present in the diets of mammal (6). In plants, the induction of Cyt P-450-dependent metabolism of xenobiotics has been reported (4, 5, 12, 15, 25), but there is no evidence that these reactions are catalyzed by enzymes exhibiting broad and overlapping substrate specificity. To the contrary, the approximately 30 Cyt P-450 monooxygenase reactions already described in plants show very narrow substrate specificity (3).

We have recently characterized a xenobiotic-inducible Cyt P-450 from wheat microsomes that catalyzes the aryl hydroxylation of diclofop, a herbicide selective for this crop (25). Here, we further report on the regiospecificity and substrate specificity of this enzyme. We show that this diclofop-metabolizing activity is similar to a new LAH[3] from wheat (26). Therefore, for the first time, we report a relationship between a Cyt P-450 oxygenase involved in a defined physiological reaction and the ability to metabolize a xenobiotic.

MATERIALS AND METHODS

Radiochemicals

Radiolabeled [1-^{14}C]lauric acid and [3-^{14}C]cinnamic acid were from Commissariat à l'Energie Atomique (Saclay, France), and [phenyl U-^{14}C]2,4-D from Sigma; [2,4-dichlorophenoxy U-^{14}C]diclofop-methyl was kindly provided by Hoechst AG. (Frankfurt am Main, Germany), [triazine-2-^{14}C] chlorsulfuron by E. I. Du Pont de Nemours and Co.Inc., and

[1] This work was supported by the Centre National de la Recherche Scientifique and by a Ministère de la Recherche et de la Technologie grant to A.Z.

[2] Present address: Department of Biochemistry, Royal Holloway and Bedford New College, University of London, Egham, Surrey TW20 OEX, U.K.

[3] Abbreviations: LAH, lauric acid hydroxylase; DIAH, diclofop aryl hydroxylase; CA4H, cinnamic acid 4-hydroxylase; CS-OX, chlorsulfuron oxidase; CTU-OX, chlortoluron oxidase; 2,4-D-OX, 2,4-D oxidase; NA, napthalic acid anhydride; PB, phenobarbital; 11-DDNA, 11-dodecenoic acid; RP-HPLC, reverse phase-HPLC; R_T, retention time; I_{50}, concentration at 50% inhibition; ECOD, ethoxycoumarin-*O*-deethylase.

[phenyl U-^{14}C]chlortoluron by the Laboratoire des Herbicides (Institut National de la Recherche Agronomique, Dijon, France).

Plant Material

Wheat seeds (*Triticum aestivum* L., cv Etoile de Choisy) were soaked for 6 h in water at room temperature and then germinated on cheesecloth at 25°C in the dark for 48 h. Approximately 40 g of etiolated seedlings were harvested and incubated for 72 h in the dark at room temperature in 1.5 L of distilled water. The incubation medium was vigorously bubbled with filtered and hydrated air (4.5 L/min).

Wheat Treatment with NA and PB

NA treatment was performed by coating the seedlings before germination (0.25%, w/w). PB was applied by incubating the seedlings in a 5 mM solution.

Preparation of Microsomes

Approximately 25 g of wheat shoots (1–2 cm) were harvested and homogenized with an Ultra-turrax (8000 rpm, 30 s) in 100 mL of 0.1 M Na-phosphate buffer (pH 7.4) containing 250 mM sucrose, 40 mM ascorbate, 15 mM 2-mercaptoethanol, and 125 mg soluble PVP (buffer A). The homogenate was filtered through 50-μm blutex cloth and centrifuged for 15 min at 10,000*g*. The resulting supernatant was centrifuged for 45 min at 100,000*g*. The microsomal pellets were resuspended in 0.1 M Na-phosphate (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% (v:v) glycerol, homogenized with a Potter-Elvehjem homogenizer, and stored at a final protein concentration of 2.0 to 3.5 mg/mL at −80°C. All enzyme extraction and centrifugation procedures were conducted at 4°C.

Enzyme Assays

DIAH was measured by following the rate of hydroxylated product formation. The standard assay contained, in a final volume of 0.25 mL, 0.14 to 0.21 mg of microsomal proteins, 0.1 M Na-phosphate (pH 7.4), 0.6 mM NADPH, 6.7 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate dehydrogenase, and 100 μM [2,4-dichlorophenoxy U-^{14}C]diclofop. The reaction was stopped after 10 min incubation at 28°C by adding 0.15 mL acetone:1N H_2SO_4 (3:1). The acid acetonic reaction mixture was extracted twice with 0.5 mL of diethyl ether and the hydroxylated reaction products analyzed by TLC (25) and RP-HPLC. A similar procedure for incubation and extraction of reaction products was used to measure LAH, CTU-OX, CS-OX, and 2,4-D-OX, replacing diclofop by [1-^{14}C]lauric acid (2.09 GBq/mmol), [phenyl U-^{14}C]chlortoluron (440 MBq/mmol), [triazine-2-^{14}C]chlorsulfuron (201.3 MBq/mmol), or [phenyl U-^{14}C]2,4-D (925 MBq/mmol) at a final concentration of 0.1 mM.

TLC separation of chlortoluron and lauric acid metabolites was as described in references 15 and 26, respectively. ECOD was assayed as in reference 24. Hydroxy-chlorsulfuron (R_F = 0.62) was separated from the substrate (R_F = 0.74) by developing TLC plates in the solvent system benzene:acetone:formic acid (30:10:1; v/v/v) (2×). Hydroxy-2,4-D (R_F = 0.43) was separated from the substrate (R_F = 0.59) by developing TLC plates in the solvent system diethyl ether:light petroleum (bp 40–60°C):formic acid (70:30:1; v/v/v). CA4H was assayed as in reference 1. When both DIAH and LAH were measured in the same assay, hydroxy diclofop (R_F = 0.33) and hydroxy lauric acid (R_F = 0.60) were separated from diclofop (R_F = 0.44) and lauric acid (R_F = 0.78) by developing TLC plates in the solvent system hexane:2-propanol:acetic acid (800:300:5; v/v/v).

Enzyme activity was maintained over several weeks in microsomes stored at −80°C. Cytochrome P-450 was measured by the method of Omura and Sato (16), assuming an absorption coefficient of 91 mM^{-1} cm^{-1} for the 448 to 490 nm absorbance difference. Microsomal protein was estimated by the Bio-Rad protein microassay procedure using BSA as standard.

RP-HPLC Measurements

RP-HPLC analyses of the microsomal extracts containing diclofop metabolites were conducted using a 15 cm × 4.6 mm Beckman Ultrasphere ODS 5-μm C18 column as described previously (20). Radioactivity of RP-HPLC effluents was quantified with a computerized on-line solid scintillation counter (Ramona-D ISOMESS, Germany). Reaction products were developed (2 mL/min) using an initial mobile phase of acetonitrile:water:acetic acid (25:73:2) during 88 min, and 30:68:2 (v/v/v) from 88 to 131 min. The (2,5-dichloro-4-hydroxyphenoxy)diclofop (R_T = 78 min) and (2,3-dichloro-4-hydroxyphenoxy)diclofop (R_T = 83 min) were separated from (2,4-dichloro-5-hydroxyphenoxy)diclofop (R_T = 111 min) and coeluted with authentic standards. Residual substrate was eluted by applying a linear gradient (0–100%) of 80% acetonitrile in 0.2% aqueous acetic acid 131 min after injection and during 15 min at the same flow rate.

Inhibition and Inactivation Studies

The standard assay contained 20 μM diclofop. The compounds to be tested, dissolved in ethanol, were added to give final concentrations from 5 to 80 μM. The apparent I_{50} were estimated using a linear regression analysis program. Each value is the mean of triplicates.

Enzyme inactivation was measured by preincubating microsomes at 28°C during 8 min with 11-DDNA (20 μM) and 1 mM NADPH. The reaction mixture was then diluted 10 times with buffer containing 110 μM [1-^{14}C]lauric acid, 44 μM [2,4-dichlorophenoxy U-^{14}C]diclofop, and 1 mM NADPH to measure residual DIAH and LAH activities during a subsequent 10-min incubation. Controls were carried out by incubating microsomes with NADPH or 11-DDNA alone during the 8-min preincubation period, and 2 μM 11-DDNA during a 10-min assay.

Alternative Substrate Kinetics

DIAH and LAH activities were measured in the same assay. The assays were conducted in standard conditions (see above) with 10 to 80 μM diclofop (A) and 5 to 20 μM lauric acid (B).

Separation of lauric acid (R_F(A) = 0.78) and diclofop (R_F = 0.44) from hydroxy-laurates (R_F = 0.60) and hydroxy-diclofop (R_F = 0.33) was achieved by developing thin-layer plates in a hexane:isopropanol:acetic acid (800:300:5; v/v/v) solvent mixture.

The data was fitted on the equation (Eq. 1) for alternative substrate kinetics (2) using a nonlinear regression program written in PASCAL and run on a IBM-PC computer. The lines in Figure 2 represent computer calculation fitting this model. The points are the means of triplicates, and for each point the calculated SD was introduced as a weight into the model.

$$V_A + V_B = (V_{maxA} \times S_A/K_{mA} + V_{maxB} \times S_B/K_{mB})/(1 + S_A/K_{mA} + B/K_{mB}) \quad (1)$$

Computer-Aided Molecular Modeling

The structure of the compounds was modeled with the SYBYL 5.3 tridimensionnal infographic analyzing system (Tripos Associates Inc., St. Louis, MO). The systematic search method followed by minimization yielded the more stable configuration of diclofop (Fig. 2). Superimposition of the carboxylic functions of diclofop and lauric acid, and of C11 of lauric acid with C4 of the 2,4-dichlorophenoxy moiety of diclofop, was imposed.

RESULTS AND DISCUSSION

Inhibition Studies

Wheat varieties that are tolerant to herbicides such as diclofop, chlorsulfuron, 2,4-D, or chlortoluron are capable of rapidly degrading them by oxidation (12, 15, 21, 25). These oxidation reactions are catalyzed by Cyt P-450 in the case of the aryloxyphenoxypropionate herbicide diclofop (12, 25) and the phenylurea herbicide chlortoluron (15). A major unanswered question is whether a single enzyme is responsible for this herbicide metabolism or whether there are separate enzymes acting on different chemicals (7). In the case of DIAH, the very low K_m of the enzyme for diclofop and the Michaelian substrate saturation kinetics described in our previous study (25) suggest that a single Cyt P-450 isozyme is involved in the reaction. To determine the substrate specificity of the wheat enzyme, we studied the interaction of structural analogs of diclofop (Fig. 1) and of other known or putative Cyt P-450 substrates with the DIAH activity (Table I).

Effect of Diclofop Acid Analogs

The methyl ester of diclofop was found to be a poor inhibitor of DIAH (Table I). This suggests that under physiological conditions a negative charge at the carboxyl end of the molecule (pK_a diclofop = 3.4) is needed for interaction at the catalytic site. Close chemical analogs of diclofop like haloxyfop and fluazifop did not inhibit DIAH activity, indicating that replacement of the chlorine at C4 (Van der Waals radius = 1.8 Å) by a larger group such as CF_3 (Van der Waals radius = 2.3 Å) abolishes substrate recognition due to steric hindrance. Since haloxyfop and fluazifop have the same mode of action as diclofop (11), the sensitivity of wheat toward these herbicides may be explained by the inability of Cyt P-450 to oxidize them. It is interesting that diclofenac, an anti-inflammatory drug inhibiting the biosynthesis of prostaglandins by competing with arachidonate (22), was found partially to inhibit DIAH activity.

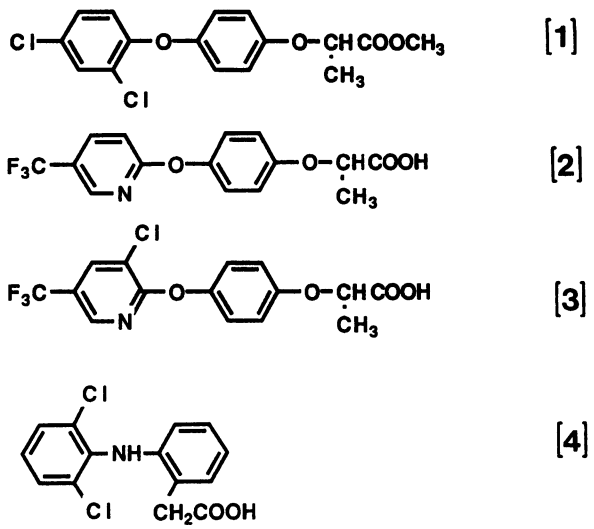

Figure 1. Chemical structures of diclofop-methyl [1], fluazifop [2], haloxyfop [3], and diclofenac [4].

Physiological Cyt P-450 Substrates

The effects of various endogenous substrates on DIAH activity are shown in Table I. Because a negative charge appeared important for substrate recognition (see above), we tested several acids known to be Cyt P-450 endogenous substrates in plants (3). Capric, lauric, myristic, and cinnamic acids can be hydroxylated in wheat (26) and a wheat Cyt P-450 enzyme (LAH) has been shown to be responsible for lauric acid hydroxylation (26). Table I shows that lauric acid is the most potent inhibitor of DIAH of all the compounds tested. A more detailed study established a K_i = 2.2 ± 0.2 μM for lauric acid as a competitive inhibitor of DIAH. Conversely, inhibition kinetics of LAH have shown that diclofop inhibited LAH competitively with a K_i of 12.7 ± 1.2 μM (26).

Putative Xenobiotic Cyt P-450 Substrates

To explore whether DIAH is implicated in the metabolism of other herbicides or whether different enzymes are acting on these compounds, we have checked the interaction of various herbicides and other xenobiotics with DIAH activity. These compounds, which are suspected on the basis of in vivo studies to be metabolized by Cyt P-450 in wheat or in other materials, appeared at best to be very weak inhibitors of DIAH (Table I), suggesting that they are metabolized by other Cyt P-450 isozymes or other types of oxidizing enzymes.

Table I. *Inhibitory Effect of Several Compounds on DIAH and LAH*

The assays were conducted in standard conditions with diclofop or lauric acid concentrations of 20 μM. The inhibitory effect on DIAH was estimated with 40, 60, and 80 μM compound concentrations (5, 10, and 20 μM for tetcyclacis, lauric acid, and capric acid). The inhibitory effect on LAH was estimated with 80, 160, and 320 μM compound concentrations. Apparent I_{50} values were determined by linear regression. Each value is the mean ± SD with $n = 3$. NI, Not inhibitory in the test conditions.

Compounds	Apparent I_{50}	
	DIAH	LAH
	μM	
Chemical analogs		
Diclofop-methyl	242 ± 24	
Fluazifop	NI	
Haloxyfop	NI	NI
Diclofenac	67 ± 14	213 ± 4
Possible Cyt P-450 endogenous substrates		
Lauric acid	10 ± 4	
Capric acid	33 ± 13	
Cinnamic acid	90 ± 16	
Myristic acid	156 ± 31	
Possible Cyt P-450 xenobiotic substrates		
2,4-D	103 ± 10	NI
Bromoxynil	148 ± 17	
Bentazone	175 ± 8	
Metribuzine	187 ± 22	
Chlortoluron	215 ± 21	NI
Chlorsulfuron	NI	
Diuron	NI	
Trifluralin	NI	
Benefin	NI	
Ethoxycoumarin	NI	

Induction Studies

Because we had previously shown that different Cyt P-450 activities may be differentially induced by exposing plants to various xenobiotics (18), we have examined the effect of treating wheat seedlings with the safener NA and with PB, a well-known Cyt P-450 inducer. The activity of DIAH, LAH, CTU-OX, CS-OX, and ECOD and the content of Cyt P-450 were enhanced in etiolated wheat shoot microsomes after treatment with either NA or PB, whereas CA4H was decreased (Table II). The safener NA was less efficient than PB in stimulating the activity of enzymes and the Cyt P-450 content (Table II). A much greater stimulation of enzyme activity occurred when PB and NA were combined. For example, the oxidation of 2,4-D, which is too low to be detected in microsomes from untreated seedlings, became measurable (Table II). Cyt P-450 was increased to 0.5 nmol/mg, one of the highest contents so far recorded in plants. Further evidence of the similarity between DIAH and LAH is provided by their induction patterns, which appeared closely parallel and more related than those of the other activities. CTU-OX was strongly enhanced by the combined treatment, whereas ECOD was barely stimulated. These results suggest that different Cyt P-450 isozymes are involved in the oxidative detoxification of herbicides in wheat.

Regiospecificity of DIAH

Wheat microsomes catalyzed predominantly the formation of three hydroxylated metabolites of (2,4-dichlorophenoxy)diclofop coeluting with authentic (2,5-dichloro-4-hydroxyphenoxy)diclofop (Fig. 2, peak A), (2,3-dichloro-4-hydroxyphenoxy)diclofop (peak B), and (2,4-dichloro-5-hydroxyphenoxy)diclofop (peak C) in RP-HPLC. These isomers can be produced from pure (2,4-dichlorophenoxy)diclofop via the formation and spontaneous rearrangement of a unique arene oxide intermediate by a mechanism known as NIH Shift (8, 23). Arene oxide formation and the resulting NIH Shift are typical of Cyt P-450-mediated aromatic hydroxylation (17). The relative amounts of the different isomers formed were 67, 24.7, and 8.3% for A, B, and C, respectively, and were found to remain constant under all induction or inhibition conditions described in this study.

This distribution differs, however, from that described in vivo by Tanaka and coworkers (A = 55%, B = 21%, and C = 24%) (23). This difference could be explained by the involvement of more than one type of enzyme or Cyt P-450 isozyme in the hydroxylation of diclofop in vivo. However, the method employed by Tanaka requires the conversion of the extracted hydroxy diclofop isomers into their methyl esters prior to their separation through RP-HPLC. We have found that the spontaneous esterification of a mixture of the pure isomers A and B in methanol produces a compound coeluting with authentic isomer C in RP-HPLC (results not

Table II. *Effect of NA, PB, and NA plus PB Treatments of Etiolated Wheat on DIAH, LAH, CA4H, CTU-OX, CS-OX, 2,4-D-OX, and ECOD Activities and Cyt P-450 Content of Wheat Shoot Microsomes*

One hundred percent activity (untreated plants) in pmol/min·mg protein were 26 ± 7 for DIAH, 55 ± 6 for LAH, 2275 ± 31 for CA4H, 5 ± 3 for CTU-OX, 9 ± 6 for CS-OX, and 6 ± 2 for ECOD; 100% Cyt P-450 content was 163 ± 10 pmol/mg protein. Results are expressed as the mean ± SD with $n = 3$.

Treatment[a]	Relative Enzyme Activities and Cyt P-450 Content							
	DIAH	LAH	CA4H	CTU-OX	CS-OX	2,4-D-OX	ECOD	Cyt P-450
H_2O	100	100	100	100	100	—	100	100
NA	403 ± 27	450 ± 24	64.7 ± 0.9	360 ± 160	900 ± 111	—	233 ± 50	154 ± 5
PB	973 ± 111	1220 ± 42	62.3 ± 1.9	812 ± 204	2377 ± 122	—	483 ± 52	201 ± 9
NA + PB	2003 ± 83	2112 ± 60	26.4 ± 0.6	3500 ± 121	3388 ± 77	100	760 ± 100	300 ± 11

[a] Treatment of seedlings was as described in "Materials and Methods."

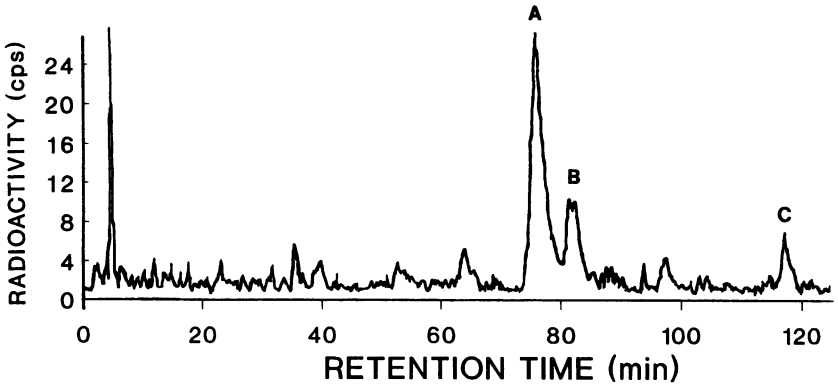

Figure 2. Reverse phase chromatography of radioactive hydroxy diclofop formed by microsomal incubations from wheat shoot with [2,4-dichlorophenoxy U-^{14}C]diclofop in standard conditions. The assigned structures of peaks A [(2,5-dichloro-4-hydroxyphenoxy)diclofop], B [(2,3-dichloro-4-hydroxyphenoxy)diclofop], and C [(2,4-dichloro-5-hydroxyphenoxy)-diclofop] were obtained by coelution with authentic standards. The conditions of RP-HPLC are described in "Materials and Methods."

shown). Therefore, the protocol used in the in vivo study would artificially enrich the isomer bulk in isomer C. It is thus not excluded that the regioselectivity described in this study reflects the activity of a unique Cyt P-450 isozyme.

Some Cyt P-450 enzymes are known to be highly regio- and stereospecific (17). Differences in specificity are often used to demonstrate the involvement of more than one Cyt P-450 isozyme in the metabolism of a compound. In plants, the effects of inducers have clearly established the existence of multiple forms of Cyt P-450 and their differential induction by xenobiotics (3). If different isoforms are implicated in the metabolism of diclofop, the inhibition of the enzyme or the treatment of wheat with different inducers could alter the regiospecificity of hydroxylation.

Identity of DIAH and LAH

As LAH and DIAH show very similar patterns of inhibition (Table I) and induction (Table II), we have further explored the similarities between these two activities by kinetic studies and enzyme inactivation studies. The structural similarity of both substrate was studied by computer-aided molecular modeling to test the possibility of both compounds fitting in the same catalytic site.

Alternative Substrate Kinetics

Equations describing the kinetics of one enzyme reacting with two alternative substrates have been developed by Cha (2). Measuring both LAH and DIAH activities in the same assay and fitting the data to this kinetic model by nonlinear regression analysis (Fig. 3) clearly indicated that both reactions may be catalyzed by the same isozyme. If, alternatively, the two compounds were substrates of different enzymes, the lines in Figure 3 would not intersect on the ordinate (9). The following kinetic parameters were determined: K_m for diclofop, 5.6 ± 1.2 μM; V_{max} for diclofop, 623 ± 27 pmol/min·mg protein; K_m for lauric acid, 8.5 ± 1.7 μM; V_{max} for lauric acid 2732 ± 240 pmol/min·mg protein; K_i for diclofop, 23.2 ± 3.9 μM; K_i for lauric acid, 0.8 ± 0.5 μM. These K_m and K_i values obtained are in good agreement with those from experiments where each substrate was taken independently (25, 26).

Enzyme Inactivation by 11-DDNA

If diclofop and lauric acid oxidation were mediated by the same Cyt P-450 isozyme, then enzyme inactivation should equally inhibit both activities. This technique has been used

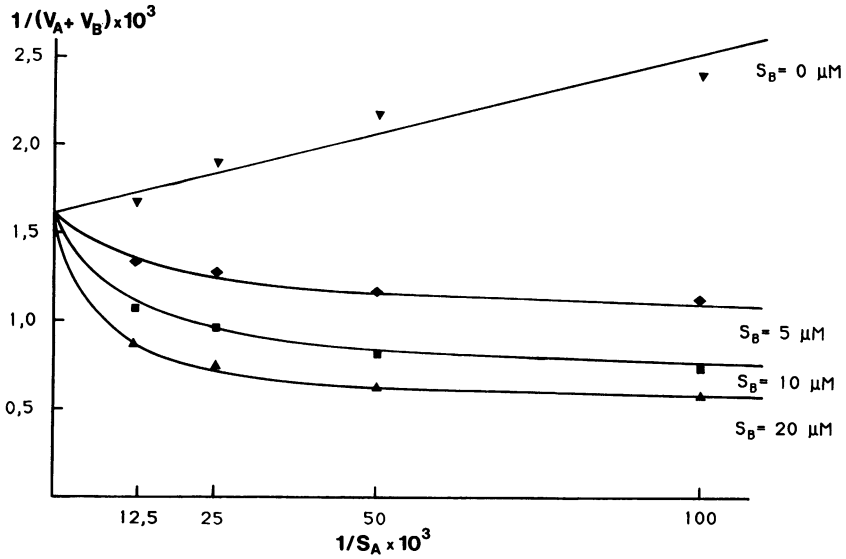

Figure 3. Alternative substrate kinetics: Effect of constant amounts of lauric acid (S_B) at varying concentrations of diclofop (S_A) on rates of hydroxylated lauric acid (V_B) and hydroxylated diclofop (V_A) formation. Wheat microsomes were incubated as described in "Materials and Methods." Kinetic parameters were estimated by fitting the data using a nonlinear regression program. Rates are expressed in pmoles/min·mg protein and concentrations in μmol/L.

previously to determine the involvement of distinct mammalian Cyt P-450 isozymes in the hydroxylation of lauric acid (17). The terminal olefin 11-DDNA is a mechanism-based inactivator of LAH in wheat (26) and in other materials (19). The loss of DIAH and LAH activities after preincubation of wheat microsomes for 8 min with NADPH or with NADPH and 11-DDNA is shown in Table III. A decrease of LAH and DIAH activities was also observed when NADPH was preincubated alone with microsomes, but the rate of inactivation was much greater in the presence of 11-DDNA. Competition of 11-DDNA with both substrates was negligible in the assay conditions. Activities of both LAH and DIAH were similarly affected in all the conditions tested.

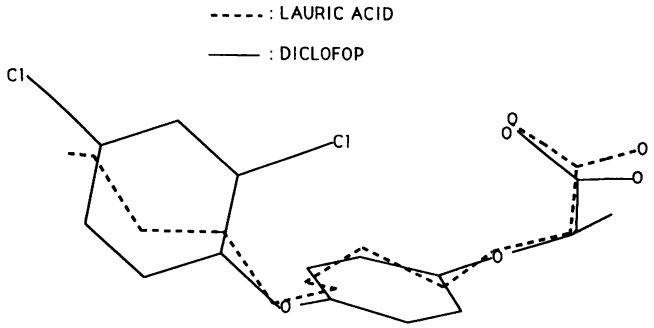

Figure 4. Structure of lauric acid superimposed over the structure of diclofop. Both rings of diclofop are aromatic.

Computer-Aided Molecular Modeling

Regiospecificity studies clearly showed that position 4 in the dichlorophenyl moiety was by far the predominant site of hydroxylation (82%) of diclofop by DIAH. Our studies also showed that the negative charged carboxyl end of diclofop is important for effective binding at the active site. Furthermore, a size constraint at the 4-chloro position is shown by the lack of inhibition observed with haloxyfop and fluazifop, two diclofop analogs sharing a CF_3 in this position (Table I). Because lauric acid is a much better inhibitor of DIAH than capric and myristic acids, it is likely that lauric acid has the optimal size for binding at the DIAH active site. Thus, taking into account these features, we have modeled the structure of diclofop and superimposed lauric acid. Figure 4 shows that the conformations and hydroxylation positions of both substrates are compatible with binding and catalysis by the active site of one isozyme. Furthermore, the terminal methyl group of lauric acid, which superimposes with the chlorine at C4 from diclofop, has a similar Van der Waals

Table III. *Autocatalytic Inactivation of DIAH and LAH by 11-DDNA*

Enzyme inactivation was measured by preincubating microsomes at 28°C during 8 min with 11-DDNA (20 μM) and 1 mM NADPH. The reaction mixture was then diluted 10 times with buffer containing 110 μM [1-^{14}C]lauric acid, 44 μM [2,4-dichlorophenoxy U-^{14}C]diclofop, and 1 mM NADPH to measure residual DIAH and LAH activities during a 10-min incubation. Hydroxy diclofop and hydroxy lauric acid were separated from diclofop and lauric acid using a TLC system described in "Materials and Methods." Controls were carried out by incubating microsomes with NADPH or 11-DDNA alone during the 8-min preincubation period, and 2 μM 11-DDNA during the 10-min assay. Data are the mean ± SD with $n = 3$. One hundred percent activity was 619 ± 3.3 and 173 ± 5.0 pmol/min·mg protein for LAH and DIAH, respectively.

Conditions			Relative Residual Activities	
Pre-incubation	Incubation		LAH	DIAH
		mM		
Microsomes	NADPH	1		
	Lauric acid	0.01	100	100
	Diclofop	0.04		
Microsomes + NADPH (1 mM)	NADPH	1		
	Lauric acid	0.01	79.3 ± 2.2	83.6 ± 1.8
	Diclofop	0.04		
Microsomes	NADPH	1		
	Lauric acid	0.01	97.5 ± 1.9	98.2 ± 2.3
	Diclofop	0.04		
	+11-DDNA	0.002		
Microsomes + 11-DDNA (0.02 mM)	NADPH	1		
	Lauric acid	0.01	88.2 ± 3.3	86.6 ± 1.2
	Diclofop	0.04		
	11-DDNA	0.002		
Microsomes + 11-DDNA (0.02 mM) + NADPH (1 mM)	NADPH	1		
	Lauric acid	0.01	56.6 ± 2.0	57.6 ± 1.1
	Diclofop	0.04		
	11-DDNA	0.002		

radius (2 Å). However, it should be emphasized that lauric acid has a very wide range of stable low energy conformations and could also be fitted to other structures.

CONCLUSION

It is commonly assumed that the animal Cyt P-450 isoforms with broad substrate specificity that metabolize foreign chemicals have appeared under the pressure of plant secondary metabolites present in their diet. In plants, it is more likely that the metabolism of xenobiotics by Cyt P-450 reactions is the result of structural similarities between the xenobiotic and physiological substrates of Cyt P-450 isozymes that serve other, still undefined roles in plant metabolism.

In the present study, we show that microsomes from NA and PB pretreated wheat seedlings actively catalyze the NADPH-dependent oxidative metabolism of chlorsulfuron, 2,4-D, chlortoluron, and ethoxycoumarin. The Cyt P-450-dependent oxidation of chlortoluron in wheat cell suspension cultures has also been described by Mougin (15). Ethoxycoumarin, a model substrate representative of the Cyt P-450 xenobiotic-metabolizing system in mammals, is dealkylated by such an enzyme in Jerusalem artichoke and wheat (24). There is evidence (to be published) that the oxidation of 2,4-D and chlorsulfuron are also catalyzed by Cyt P-450. The different patterns of induction and inhibition of these activities is indicative that several distinct Cyt P-450 species are involved in these reactions.

The hydroxylation of diclofop appears to be mediated by a single Cyt P-450 isoform with narrow substrate specificity because (a) the activity exhibits Michaelian kinetics, (b) several induction and inhibition conditions do not modify the ratio of the three isomeric reaction products, and (c) with the exception of lauric acid, none of the known or supposed substrates of the wheat-oxidizing system interfered with DIAH activity.

Although definite proof of the identity of LAH and DIAH may only be provided by studies with purified enzyme fractions, our results strongly suggest that DIAH is identical to a new lauric acid (ω-1)-hydroxylase that we recently characterized in wheat microsomes: (a) both activities have similar K_m values for lauric acid and diclofop and these compounds competitively inhibit the hydroxylation of each other with nearly identical K_i values; (b) they are induced by the same inducers with similar kinetics; (c) 11-DDNA, a suicide-substrate of laurate hydroxylases, produced comparable inactivation of both activities; (d) the reaction kinetics in presence of both substrates are consistent with an interaction on a single enzyme; and (e) both substrates may assume low energy conformations compatible with their binding to and oxidation by a single enzyme. It is interesting to note that microsomes from other organisms we tested (rat liver, fish liver, and *Drosophila*) that carry (ω-1)-LAH activity are able to metabolize diclofop (to be published). The level of DIAH activity in these microsome preparations was closely related to the (ω-1)-LAH activity level. Furthermore, plants that do not exhibit (ω-1)-LAH activity do not metabolize diclofop despite high LAH activities. Kobek (10) has recently shown that metabolism through aryl hydroxylation is the basis of diclofop resistance in red fescue (*Festuca rubra*). We performed GC-MS studies that show that red fescue microsomes hydroxylate lauric acid at C11 (to be published).

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. S.D. Frear for the generous gift of authentic hydroxylated diclofop analogs. They also wish to thank Dr. S. Powles for valuable discussions and Dr. J. Jaganathan for performing the molecular modeling studies.

LITERATURE CITED

1. **Benveniste I, Salaün JP, Durst F** (1977) Wounding-induced cinnamic acid hydroxylase in Jerusalem artichoke tuber. Phytochemistry **16:** 69–73
2. **Cha S** (1968) Kinetics of enzyme reactions with competing alternative substrates. Mol Pharmacol **4:** 621–629
3. **Durst F** (1991) Biochemistry and physiology of plant cytochrome P-450. *In* K Ruckpaul, H Rein, eds, Microbial and Plant Cytochrome P-450: Biochemical Characteristics, Genetic Engineering and Practical Implications, Frontiers in Biotransformation. Vol. 4. Akademie Verlag, Berlin, pp 191–232
4. **Fonne-Pfister R, Gaudin J, Kreuz K, Ramsteiner K, Ebert E** (1990) Hydroxylation of primisulfuron by an inducible cytochrome P-450-dependent monooxygenase system from maize. Pestic Biochem Physiol **37:** 165–173
5. **Fonne-Pfister R, Kreuz K** (1990) Ring-methyl hydroxylation of chlortoluron by an inducible cytochrome P-450-dependent enzyme from maize. Phytochemistry **29:** 2793–2796
6. **Gonzalez FJ, Nebert DW** (1990) Evolution of the P450 gene superfamily: Animal-plant "warfare", molecular drive and human genetic differences in drug oxidation. Trends Genet **6:** 182–186
7. **Gressel J** (1988) Multiple resistances to wheat selective herbicides: new challenges to molecular biology. *In* BJ Miflin, ed, News and Views: Oxford Surveys of Plant Molecular and Cell Biology. Vol 5. Oxford University Press, Oxford, England, pp 195–203
8. **Guroff G, Daly JW, Jerina DM, Renson J, Witkop B, Udenfriend S** (1967) Hydroxylation-induced migration: The NIH Shift. Science **157:** 1524–1530
9. **Jeffery EH, Mannering GJ** (1983) Interaction of constitutive and phenobarbital-induced cytochrome P-450 isozymes during the sequential oxidation of benzphetamine. Explanation for the difference in benzphetamine-induced hydrogen peroxide formation and 455 nm complex formation in microsomes from untreated and phenobarbital-treated rats. Mol Pharmacol **23:** 748–757
10. **Kobek KH** (1990) Untersuchungen über wirkungsmechanismus und selektivität von inhibitoren und herbiziden der de novo fettsäurebiosynthese. Karlsruhe Beiträge Pflanzenphysiol **18:** 1–134
11. **Lichtenthaler HK** (1990) Mode of action of herbicides affecting acetyl-CoA-carboxylase and fatty acid biosynthesis. Z Naturforsch **45c:** 521–528
12. **McFadden JJ, Frear DS, Mansager ER** (1989) Aryl hydroxylation of diclofop by a cytochrome P-450 dependent monooxygenase from wheat. Pestic Biochem Physiol **34:** 92–100
13. **McFadden JJ, Gronwald JW, Eberlein CV** (1990) In vitro hydroxylation of bentazon by microsomes from naphthalic anhydride-treated corn shoots. Biochem Biophys Res Commun **168:** 206–213
14. **Moreland DE, Corbin FT, Novitzky WP** (1990) Metabolism of metolachlor by a microsomal fraction isolated from grain sorghum (*Sorghum bicolor*) shoots. Z Naturforsch **45c:** 127–133
15. **Mougin C, Cabanne F, Canivenc MC, Scalla R** (1990) Hydroxylation and N-demethylation of chlortoluron by wheat microsomal enzymes. Plant Sci **66:** 195–203
16. **Omura T, Sato R** (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. II.

Solubilization, purification and properties. J Biol Chem **239:** 2379–2385

17. **Ortiz de Montellano PR** (1986) Cytochrome P-450: Structure, Mechanism and Biochemistry. New York, Plenum Press, pp 217–271
18. **Reichhart D, Salaün JP, Benveniste I, Durst F** (1979) Induction by manganese, ethanol, phenobarbital and herbicides of microsomal cytochrome P-450 in higher plant tissues. Arch Biochem Biophys **196:** 301–303
19. **Salaün J-P, Reichhart D, Simon A, Durst F, Reich NO, Ortiz de Montellano PR** (1984) Autocatalytic inactivation of plant cytochrome P-450: Selective inactivation of the lauric acid in-chain hydroxylase from *Helianthus tuberosus* L. by unsaturated substrate analogs. Arch Biochem Biophys **232:** 1–7
20. **Salaün JP, Simon A, Durst F** (1986) Specific induction of lauric acid ω-hydroxylase by clofibrate, diethylhexyl-phtalate and 2,4-dichlorophenoxy-acetic acid in higher plants. Lipids **21:** 776–779
21. **Sheel D, Sandermann H Jr** (1981) Metabolism of 2,4-dichlorophenoxyacetic acid in cell suspension cultures of soybean (*Glycine max* L.) and wheat (*Triticum aestivum* L.). Planta **152:** 248–252
22. **Small RE** (1989) Diclofenac sodium. Clin Pharm **8:** 545–558
23. **Tanaka FS, Hoffer BL, Shimabukuro RH, Wien RG, Walsh WC** (1990) Identification of the isomeric hydroxylated metabolites of methyl-2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate (diclofop-methyl) in wheat. J Agric Food Chem **38:** 559–565
24. **Werck-Reichhart D, Gabriac B, Teutsch H, Durst F** (1990) Two cytochrome P-450 isoforms catalysing O-deethylation of ethoxycoumarin and ethoxyresorufin in higher plants. Biochem J **270:** 729–735
25. **Zimmerlin A, Durst F** (1990) Xenobiotic metabolism in plants: Aryl hydroxylation of diclofop by a cytochrome P-450 enzyme from wheat. Phytochem **29:** 1729–1732
26. **Zimmerlin A, Salaün J-P, Durst F, Mioskowski C** (1992) Cytochrome P-450 dependent hydroxylation of lauric acid at the subterminal position and oxidation of unsaturated analogs in wheat microsomes. Plant Physiol **100:** 868–873