Plant Physiol. (1995) 109: 1483–1490

Differential Induction of Cytochrome P450-Mediated Triasulfuron Metabolism by Naphthalic Anhydride and Triasulfuron[1]

Michael W. Persans and Mary A. Schuler*

Department of Plant Biology, 190 Plant and Animal Biotechnology Laboratory, University of Illinois, Urbana, Illinois 61801

Cytochrome P450 monooxygenases play paramount roles in the detoxification of herbicides as well as in the synthesis of lignins, flavonoids, and phenolic acids. Biochemical analysis of triasulfuron metabolism in maize (*Zea mays*) seedlings has demonstrated that the P450(s) responsible for detoxification of this herbicide is induced by naphthalic anhydride (NA), a plant safener, and by triasulfuron, the herbicide itself. Induction studies conducted with seedlings of different ages suggest that two separate response pathways modulate this P-450 activity. Induction by NA is independent of the developmental age of the seedlings up to 6.5 d; induction by triasulfuron is tightly modulated with respect to developmental age in that triasulfuron metabolism can be induced by triasulfuron in young (2.5 d) but not older (6.5 d) seedlings. Induction by NA administered in combination with triasulfuron synergistically enhances triasulfuron metabolism in younger seedlings to levels substantially above that obtained with either herbicide or safener treatment alone. In older seedlings, NA plus triasulfuron treatment induces triasulfuron metabolism to only the level of NA treatment alone, indicating again that the induction cascade responding to triasulfuron is nonfunctional in later development. $MnCl_2$ studies indicate that the triasulfuron insensitivity of older seedlings does not result from a general limitation in the inducibility of this P-450 detoxification system but rather from specific limitations in the triasulfuron-response pathway.

In conjunction with NADPH-dependent P-450 reductases, Cyt P450 monooxygenases (referred to as P450s) reductively cleave molecular dioxygen to produce functionalized organic substrates. In plants, P450s have been implicated in the biosynthesis of lignins, terpenoids, alkaloids, and a variety of other plant secondary compounds, as well as the detoxification of herbicides (reviews: Donaldson and Luster, 1991; Durst et al., 1992). For a number of herbicides, resistance in tolerant plants is known to be mediated by the rapid conversion of the herbicide into hydroxylated, inactive products (Frear et al., 1991) that are subsequently conjugated to carbohydrate moieties in the plant cell wall (Lamoureux and Rusness, 1986). In many of these cases, the involvement of P450s in the initial steps of herbicide detoxification was originally inferred from in vivo analysis of tolerant versus susceptible biotype plants (Christopher et al., 1991; Cotterman and Saari, 1992). More recently, definitive in vitro P450-mediated metabolism of herbicides has been demonstrated for the *N*-demethylation and ring methyl hydroxylations of chlortoluron (a phenylurea herbicide) in wheat (Mougin et al., 1990) and maize (Fonne-Pfister and Kreuz, 1990), the aryl hydroxylations of diclofop in wheat (McFadden et al., 1989; Zimmerlin and Durst, 1990, 1992), the aryl hydroxylation of triasulfuron and chlorsulfuron (sulfonylurea herbicides) in wheat (Frear et al., 1991; Thalacker et al., 1994) and maize (Moreland et al., 1993a), and the aryl- and pyrimidine-ring hydroxylations of primisulfuron (a sulfonylurea herbicide) in maize (Fonne-Pfister et al., 1990).

For several of the herbicide-metabolizing P450s mentioned above, substantial evidence indicates that a variety of compounds, as divergent as NA (a plant safener), phenobarbital, ethanol, manganese ions, and even the herbicides themselves, can induce particular P450 activities in seedling tissues. Independently, phenobarbital, ethanol, and NA increase diclofop aryl hydroxylase, chlortoluron hydroxylase, chlorsulfuron hydroxylase, and triasulfuron hydroxylase activities in wheat seedlings (Frear et al., 1991; Zimmerlin and Durst, 1992; Zimmerlin et al., 1992). Endogenous lauric acid hydroxylase activity, but not *t*-CAH activity, is also enhanced in response to these chemical inducers. In combination, ethanol and NA additively enhance three of the herbicide-metabolizing activities mentioned above (diclofop, triasulfuron, and chlorsulfuron) (Frear et al., 1991; Thalacker et al., 1994). In combination, phenobarbital and NA synergistically enhance diclofop aryl hydroxylase, chlorsulfuron hydroxylase, chlortoluron hydroxylase, ethoxycoumarin-*O*-deethylase, and lauric acid hydroxylase activities in wheat seedlings; in contrast,

[1] This work was supported by Competitive Research grant No. 94–37301–7748 from the U.S. Department of Agriculture to M.A.S. and a National Institutes of Health Predoctoral Traineeship to M.W.P.

* Corresponding author; e-mail maryschu@uiuc.edu; fax 1–217–244–1336.

Abbreviations: NA, naphthalic anhydride; NAA, 1-naphthalene acetic acid; NDA, 2,3-naphthalene dicarboxylic acid; NDiA, 1,4,5,8-naphthalenetetracarboxylic dianhydride; NI, 1,8-naphthalimide; K_s, substrate-binding coefficient; *t*-CAH, *t*-cinnamic acid hydroxylase.

t-CAH activity is synergistically repressed by this combination of chemicals (Zimmerlin and Durst, 1992; Zimmerlin et al., 1992). To our knowledge, cooperative induction effects (additive or synergistic) between herbicides and plant safeners have not yet been reported for any P450 system.

The mechanism by which NA and other crop safeners induce P450-mediated herbicide metabolism is not clear. It is already apparent that induction of herbicide metabolic activities does not result from a general stimulation of overall P450 content but rather from increased expression of individual P450s (Frear et al., 1991; Zimmerlin and Durst, 1992; Zimmerlin et al., 1992). Structural similarities between NA and NAA have suggested that this particular safener might activate developmental and tissue-specific signaling pathways that normally respond to plant hormones. In support of this theory, there is a limited amount of data suggesting that safener induction of herbicide metabolism might be developmentally regulated; for example, young wheat seedlings can be more effectively induced by NA plus ethanol treatment than can older seedlings (Thalacker et al., 1994). The observation that shoots metabolize chlorsulfuron more effectively than roots (Cotterman and Saari, 1992) suggests that herbicide metabolism may be tissue specific in some cases. Additive and cooperative interactions existing between these types of seedling-specific cues and the chemical inducers mentioned above have not yet been defined in detail.

Although one recent, intriguing study has suggested that the endogenous fatty acid lauric acid and the exogenous herbicide diclofop are hydroxylated by the same P450 (Zimmerlin and Durst, 1992), herbicide-metabolizing P450s have not yet been characterized beyond the level of metabolic and spectral binding analysis. Full-length plant P450 cDNAs have, in fact, been cloned in only a few cases, including a CYP71 sequence encoding *p*-chloro-*N*-methylaniline demethylase from avocado (Bozak et al., 1990, 1992), CYP73 sequences encoding *t*-CAH from Jerusalem artichoke (Teutsch et al., 1993), mung bean (Mizutani et al., 1993), alfalfa (Fahrendorf and Dixon, 1993), pea (M. Frank, J.M. Deyneka, and M.A. Schuler, unpublished data), a CYP72 sequence encoding a potential geraniol hydroxylase from *Catharanthus roseus* (Vetter et al., 1992), and a CYP75 sequence encoding flavonoid 3',5'-hydroxylase from petunia (Holton et al., 1993). Since the substrate reactivities for only a few of these P450s have been defined by heterologous expression in yeast (avocado CYP71 [Bozak et al., 1992], Jerusalem artichoke and alfalfa CYP73 [Fahrendorf and Dixon, 1993; Urban et al., 1994], petunia CYP75 [Holton et al., 1993]), the breadth of substrates capable of being metabolized by these and other endogenous P450s and the relatedness of herbicide-metabolizing P450s to endogenous P450s remain to be elucidated.

To determine the level of synergistic interaction among safeners, herbicides, and developmental parameters, we have characterized the level of triasulfuron metabolism in response to NA, some of its structural analogs, and triasulfuron itself during different stages in maize (*Zea mays*) seedling development. The patterns of triasulfuron metabolism suggest that two separate induction pathways modulate expression of this particular P450 activity in maize.

MATERIALS AND METHODS

Seed Growth and Herbicide Application

Approximately 100 g of captan-treated corn seeds (*Zea mays* var B73) were soaked overnight in a 1-L flask with flowing tap water. Untreated seeds (not treated with captan) were sterilized for 45 min using a 30% (v/v) bleach solution containing 0.05% (v/v) Tween 20 and washed four times with 400 mL of sterile distilled water. For most of the inducer treatments, 100 g of seeds were coated with the inducer by shaking the seeds vigorously in a 100-mL bottle with 1 g of dry powdered inducer. For induction with NAA, the seeds were soaked in a 20-μg/mL NAA solution for 15 min. Seeds were then arranged in rows on sterile white Teri towels and moistened with sterile distilled water, 20 μg/mL NAA, or 25 mM $MnCl_2$ as appropriate. The paper towels were subsequently sandwiched between cafeteria trays and placed upright in a tub of distilled water. The seeds were grown at 25 to 30°C for 2.5, 4.5, or 6.5 d. Approximately 16 h before harvesting, the seedlings were treated with triasulfuron by soaking the paper towel with 25 mL of a solution containing 0.052% (w/v) of a 75% (w/w) powder of the commercial form of triasulfuron (Amber, provided by CIBA-Geigy, Research Triangle Park, NC) corresponding to a final concentration of 1 mM triasulfuron.

Microsomal Protein Isolation

Microsomes were isolated at 4°C by mincing 5 to 10 g of fresh seedling tissue in a chilled mortar and adding 20 to 30 mL of microsomal isolation buffer (0.1 M Tris-HCl [pH 8.0], 0.5 M Suc, 1 mM EDTA, 20 mM sodium ascorbate, 1 mM DTT, 1.25 mg/mL insoluble PVP, 10% glycerol, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin). The tissue was ground to a fine pulp, filtered through four layers of sterile cheesecloth, and centrifuged for 10 min at 13,000*g* to pellet mitochondria, plastids, and cellular debris. The 13,000*g* supernatant was transferred to polycarbonate ultracentrifuge tubes, and microsomes were pelleted for 90 min at 150,000*g*. The microsomal pellet was resuspended in 0.5 to 1 mL of microsomal storage buffer (0.01 M KPO_4 [pH 7.4], 1 mM EDTA, 20% glycerol, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 5 μg/mL aprotinin) with a Dounce tissue homogenizer and used immediately for metabolism assays or binding spectra or stored frozen at −80°C. This procedure yields approximately 1 to 1.5 mg of microsomal protein g^{-1} fresh weight.

Metabolism Assays

For each metabolism assay, 1.5 to 2 mg of microsomal protein (in 200 μL) were added to an equal volume of 2× microsomal reaction mixture (0.01 M KPO_4 [pH 7.4], 1 mM EDTA, 10 mM NADPH, 20 mM Glc-6-P, 0.02% Tween 80, 2 μM FMN, 2 μM FAD). One unit of Glc-6-P dehydrogenase and 100 nmol of triasulfuron (CIBA-Geigy) in acetone or

100 nmol of *t*-cinnamic acid (Aldrich; 250 μM final substrate concentration) in acetone (0.05% final acetone concentration) were added to initiate the reaction. Reactions were incubated at 30°C for 45 min on a rocker platform (30 cycles/min) and all metabolism assays were terminated by adding 100 μL of acetone (Optima grade) and 25 μL of 4.4 M H_3PO_4 or 25 μL of acetic acid. Substrates and products were subsequently extracted four times with 1 mL of ethyl acetate (Optima grade, Fisher Scientific). The organic phases were evaporated to dryness under a stream of air, resuspended in 150 μL (for triasulfuron assays) or 300 μL (for *t*-CAH assays) of methanol (Optima grade), and stored in the dark at −20°C until analyzed by HPLC.

HPLC Analysis

After resuspension in methanol, the samples were filtered through a 0.22-μm syringe filter and 20-μL aliquots were injected into a Bio-Rad series 800 gradient HPLC system. Triasulfuron and *t*-cinnamic acid metabolites were separated on a 4.6-mm × 25-cm C_{18} column (Rainin, Woburn, MA) using a 20 to 100% linear gradient of acetonitrile in distilled water, adjusted to pH 3.0 with acetic acid. The gradient was run over a 20-min period at a flow rate of 1 mL/min. Metabolites were detected by monitoring the column eluant at 240 nm for triasulfuron metabolites and 290 nm for *t*-cinnamic acid metabolites. Between runs the HPLC column was flushed with 100% acetonitrile for 20 min and preequilibrated with 20% acetonitrile/80% water for 20 min. The metabolites of each reaction were positively identified by co-chromatography with 5-hydroxytriasulfuron (provided by CIBA-Geigy) and *p*-coumaric acid (Aldrich).

Binding Spectra

Type I-binding spectra assays were performed as described by Jefcoate (1978). Frozen microsomal protein (2–2.5 mg) from 2.5-d-old seedlings was incubated for 5 min at room temperature with 75 to 200 μM triasulfuron dissolved in microsomal storage buffer. The reference cuvette contained microsomal storage buffer plus herbicide in one chamber of the split cuvette and protein alone in the other chamber. The sample cuvette contained buffer alone in one chamber of the split cuvette and protein plus herbicide in the other. The samples were scanned from 380 to 460 nm using a Cary 219 (Varian, Harbor City, CA) spectrophotometer. The magnitude of the binding spectra is defined as $\Delta A_{(410-425)}$ mg^{-1} protein. For K_s determinations, the assays were performed as described above except that the microsomal protein was successively titrated with increasing amounts of triasulfuron (in 50 μM increments) until the triasulfuron reached 250 μM in the assay mixture. CO difference spectra and P450 quantitation were performed as described by Omura and Sato (1964) using an extinction coefficient for P450 of 91 mM^{-1} cm^{-1} for the wavelength pair (450 and 490 nm).

RESULTS

Characterization of Triasulfuron Metabolism

In this study, in vitro assays for triasulfuron (Fig. 1) and *t*-cinnamic acid metabolism in maize seedlings were conducted using isolated microsomes as described in "Materials and Methods." Because previous in vitro triasulfuron metabolism assays measured [^{14}C]hydroxytriasulfuron production by TLC analysis (Moreland et al., 1993a), the P450-mediated conversion of triasulfuron to hydroxytriasulfuron measured by HPLC analysis in our reactions was verified by demonstrating that NADPH depletion inhibited 95% of the detectable metabolism and that tetcyclasis, a plant growth regulator and P450-specific inhibitor (Frear et al., 1991), inhibited 90% of the triasulfuron metabolism (not shown). The triasulfuron metabolite 5-hydroxytriasulfuron (Fig. 1) was positively identified by co-chromatography of

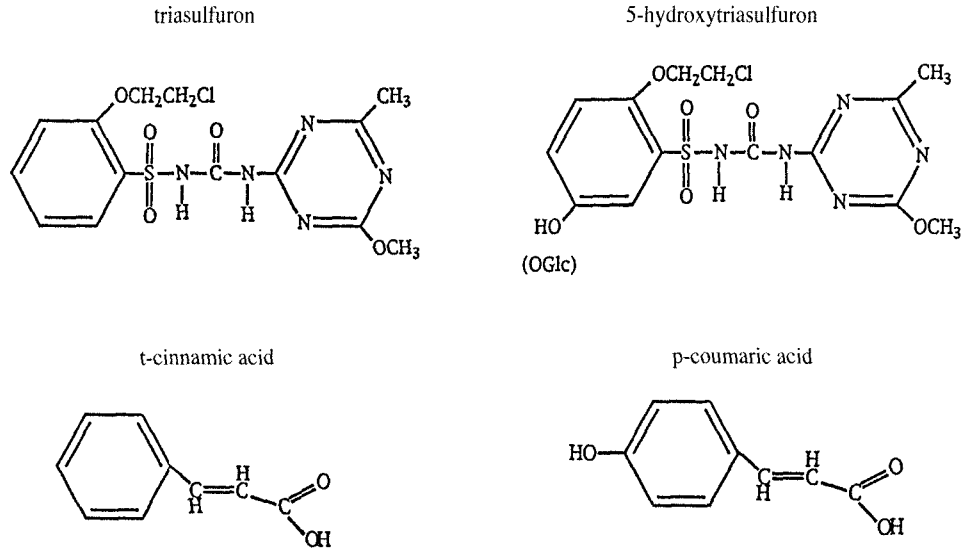

Figure 1. Substrates and metabolites for triasulfuron hydroxylase and *t*-CAH.

the 45-min in vitro reaction products with known standards in comparison with 0-min in vitro control reactions (not shown). This hydroxylated product corresponds to the same metabolite generated in vitro with wheat microsomes and positively identified by MS by Frear et al. (1991).

Induction of Triasulfuron Metabolism

To determine the level to which triasulfuron metabolism is induced by safeners and herbicides, the turnover rates for triasulfuron were monitored after induction with NA and triasulfuron separately and in combination with one another. In NA-treated 2.5- and 6.5-d-old maize seedlings, triasulfuron metabolism was induced to the same level, at least 33-fold over the level present in control seedlings (Table I). (In these calculations, we estimated the control values to be below the 10 pmol h^{-1} mg^{-1} protein detection limit of our HPLC assay system. By TLC analysis, Moreland et al. [1993a] estimated triasulfuron hydroxylase activity at approximately 20 pmol h^{-1} mg^{-1} protein in control maize seedlings.) NA maximally induced triasulfuron metabolism at a dosage of 1 to 2 g/100 g of seed and began to inhibit triasulfuron metabolism at a dosage of 3 g/100 g of seed (not shown), presumably because of the growth retardation effects incurred at high NA concentrations.

Treatment with triasulfuron alone resulted in low metabolism (78 pmol h^{-1} mg^{-1} protein; 7.8-fold induction) in 2.5-d-old seedlings and no detectable activity in 6.5-d-old seedlings (Table I). In contrast, treatment of seedlings with NA plus triasulfuron for 16 h before harvesting enhanced triasulfuron metabolism to 885 pmol h^{-1} mg^{-1} protein. This 88-fold induction, which is 2.6-fold higher than the level obtained in 2.5-d-old seedlings treated with NA alone, represents the highest level of triasulfuron metabolism obtained with any set of inducers tested in this study. At 327 pmol h^{-1} mg^{-1} protein, the level of triasulfuron metabolism in 6.5-d-old NA plus triasulfuron-treated seedlings corresponded to the level detected in 2.5- and 6.5-d-old seedlings treated with NA alone. NA plus triasulfuron-treated seedlings (4.5 d old) exhibited an intermediate level of triasulfuron metabolism (663 pmol h^{-1} mg^{-1} protein; not shown), indicating that there is a strong developmental reduction in the inducibility of this P450 system.

To determine whether other potential inducers synergistically increase triasulfuron metabolism in young seedlings, maize seedlings were treated with the inducers shown in Figure 2 in combination with triasulfuron. Several points can be made from the metabolic analyses in Table II. First, in combination with triasulfuron, all of these naphthalene derivatives stimulated triasulfuron metabolism above that obtained for 2.5-d-old seedlings (78 pmol h^{-1} mg^{-1} protein) or 6.5-d-old seedlings (undetectable) treated with triasulfuron alone. Naphthalene itself failed to induce triasulfuron metabolism when applied to 6.5-d-old seedlings in combination with triasulfuron. Second, in combination with triasulfuron, the effectiveness of this set of naphthalene derivatives varied significantly in younger seedlings. NDA plus triasulfuron treatment enhanced metabolism 5.3-fold over triasulfuron treatment alone; NI plus triasulfuron treatment increased metabolism 2.0-fold; NDiA plus triasulfuron treatment increased metabolism 1.8-fold; NAA plus triasulfuron treatment increased metabolism a mere 1.4-fold. Third, none of these treatments induced triasulfuron metabolism to a level as high as that obtained with NA plus triasulfuron treatment in either 2.5- or 6.5-d-old seedlings (88-fold versus 33-fold, respectively). Fourth, except for NDA plus triasulfuron treatment, the levels of triasulfuron metabolism achieved for these inducer plus triasulfuron treatments were statistically the same in 2.5- versus 6.5-d-old seedlings. In NDA plus triasulfuron-treated seedlings, triasulfuron metabolism was 1.9-fold higher in the younger 2.5-d-old seedlings. The high metabolic activity of 415 pmol h^{-1} mg^{-1} protein in these NDA plus triasulfuron-treated seedlings was intermediate between that achieved in 2.5-d-old NA and NA plus triasulfuron-treated seedlings (Table I).

Table I. *Induction of triasulfuron metabolism in maize seedlings (var B73)*

Inducer (g/100 g seeds)	Triasulfuron Metabolism[a]	
	2.5 d	6.5 d
	pmol h^{-1} mg^{-1} protein	
Control (0)	<10[b]	<10[b]
NA (1.0)	339.2 ± 13.4	334.3 ± 96.5
NA + triasulfuron (1.0 + 0.013)	885.0 ± 84.1	327.2 ± 19.1
Triasulfuron (0.013)	78.3 ± 23.4	<10[b]

[a] Each value is derived from two independent isolates done in duplicate. [b] HPLC detection limit = 10 pmol h^{-1} mg^{-1} protein.

Several studies have indicated that $MnCl_2$ induces P450 expression in artichoke tubers (Reichhart et al., 1980; Werck-Reichhart et al., 1993) and in common sage (Funk and Croteau, 1993). By itself, $MnCl_2$ induced a low level of triasulfuron metabolism in 6.5-d-old seedlings (Table II). In combination with NA plus triasulfuron treatment, $MnCl_2$ induced triasulfuron metabolism to 809 pmol h^{-1} mg^{-1} protein in 2.5-d-old seedlings, which is equivalent to the level attained in seedlings treated with NA plus triasulfuron without $MnCl_2$. In contrast, $MnCl_2$ induced triasulfuron metabolism from 327 to 515 pmol h^{-1} mg^{-1} protein in 6.5-d-old seedlings treated with NA, triasulfuron, and $MnCl_2$.

A prominent and widespread P450 activity detected in plants is that of *t*-CAH, which catalyzes the second common step in the phenylpropanoid pathway, the conversion of *t*-cinnamic acid to *p*-coumaric acid (Fig. 1). In contrast to the P450 isozyme(s) responsible for triasulfuron metabolism, *t*-CAH was not induced in response to treatment with NA or triasulfuron treatment or any combination thereof (Table III). Also in contrast, the activity of *t*-CAH was slightly induced (1.3-fold) in older 6.5-d-old seedlings compared to the 2.5-d-old seedlings. Conversion of *t*-cinnamic acid to *o*-coumaric acid, a minor reaction occurring in these microsomal preparations (2.14 nmol h^{-1} mg^{-1} protein), was slightly suppressed by treatment with either NA, NA plus triasulfuron, or triasulfuron alone (not shown).

Triasulfuron-Binding Spectra

To determine whether triasulfuron interacts with a constitutive P450 and/or an NA-inducible P450, associations

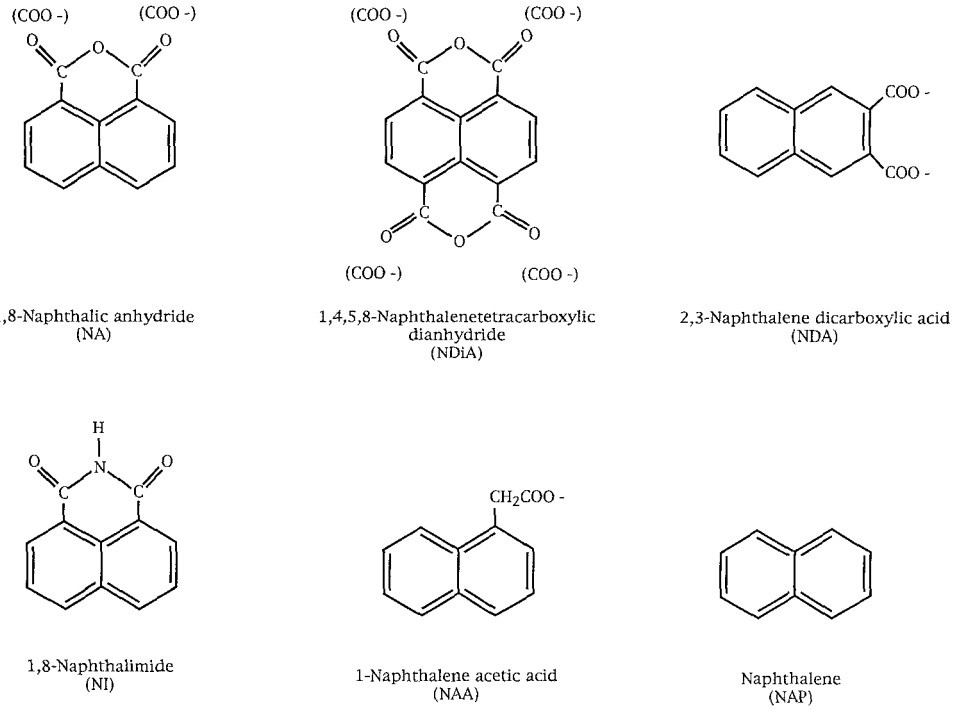

Figure 2. Structures for naphthalene derivatives used as inducers.

between triasulfuron and microsomal P450s were evaluated using substrate-binding assays as described in "Materials and Methods." Microsomes obtained from NA and NA plus triasulfuron-treated 2.5-d-old seedlings exhibited modified type I-binding spectra (Fig. 3) that are characteristic of some P450s. The magnitude of the type I-binding spectra obtained with microsomes from 2.5-d-old NA plus triasulfuron-treated seedlings was approximately 3.2-fold higher than that obtained with microsomes from 2.5-d-old NA-treated seedlings (Fig. 3). Triasulfuron did not bind at any detectable level to the microsomal P450s present in control seedlings (Fig. 3, left), indicating strong induction of a P450 not normally expressed at a detectable level in control seedlings.

To determine whether the P450 isozymes expressed in response to NA treatment versus NA plus triasulfuron treatment have similar biochemical parameters, the K_s values were defined using increasing concentrations of triasulfuron and microsomes obtained from either NA- or NA plus triasulfuron-treated seedlings. As shown in Figure 4, the K_s for triasulfuron of NA plus triasulfuron-treated microsomes was 548 ± 34 μM and the K_s for triasulfuron of NA-treated microsomes was 123 ± 0.2 μM. The P450 content of control, NA-, and NA plus triasulfuron-treated microsomes used in these assays did not vary significantly, corresponding to approximately 40 pmol P450 mg^{-1} protein. The fact that the K_s varied between the NA- and NA plus triasulfuron-treated microsomes suggests that multiple P450 isozymes(s) having different substrate binding

Table II. *Induction of triasulfuron metabolism by other naphthalene derivatives*

Inducer (g/100 g seeds)	T[a]	NA[b]	Triasulfuron Metabolism[c]	
			2.5 d	6.5 d
			pmol h^{-1} mg^{-1} protein	
NDA (1.0)	+	−	415.8 ± 60.8	221.0 ± 17.4
NDiA (1.0)	+	−	141.3 ± 17.1	168.5 ± 42.3
NI (1.0)	+	−	155.9 ± 20.3	133.9 ± 32.3
NAA (0.002)	+	−	105.9 ± 8.1	83.5 ± 19.2
Naphthalene (1.0)	+	−	ND[e]	<10[d]
25 mM $MnCl_2$	+	+	809.8 ± 66.9	515.9 ± 64.0
25 mM $MnCl_2$	−	−	ND[e]	82.9 ± 18.8

[a] + indicates addition of 0.013 g triasulfuron/100 g seed.

[b] + indicates addition of 1 g of NA/100 g of seed. [c] Each value is derived from two independent isolates done in duplicate.

[d] HPLC detection limit = 10 pmol h^{-1} mg^{-1} protein. [e] ND, Not done.

Table III. *Metabolism of t-cinnamic acid to p-coumaric acid in etiolated shoots*

Inducer (g/100 g seeds)	*t*-Cinnamic Acid Metabolism[a]	
	2.5 d	6.5 d
	nmol h^{-1} mg^{-1} protein	
Control (0)	68.6 ± 1.9	85.6 ± 4.3
NA (1.0)	65.3 ± 5.5	86.7 ± 7.1
NA + triasulfuron (1.0 + 0.013)	68.8 ± 5.9	93.7 ± 4.5
Triasulfuron (0.013)	63.7 ± 18.6	76.7 ± 7.0

[a] Each value was derived from two independent isolates done in duplicate.

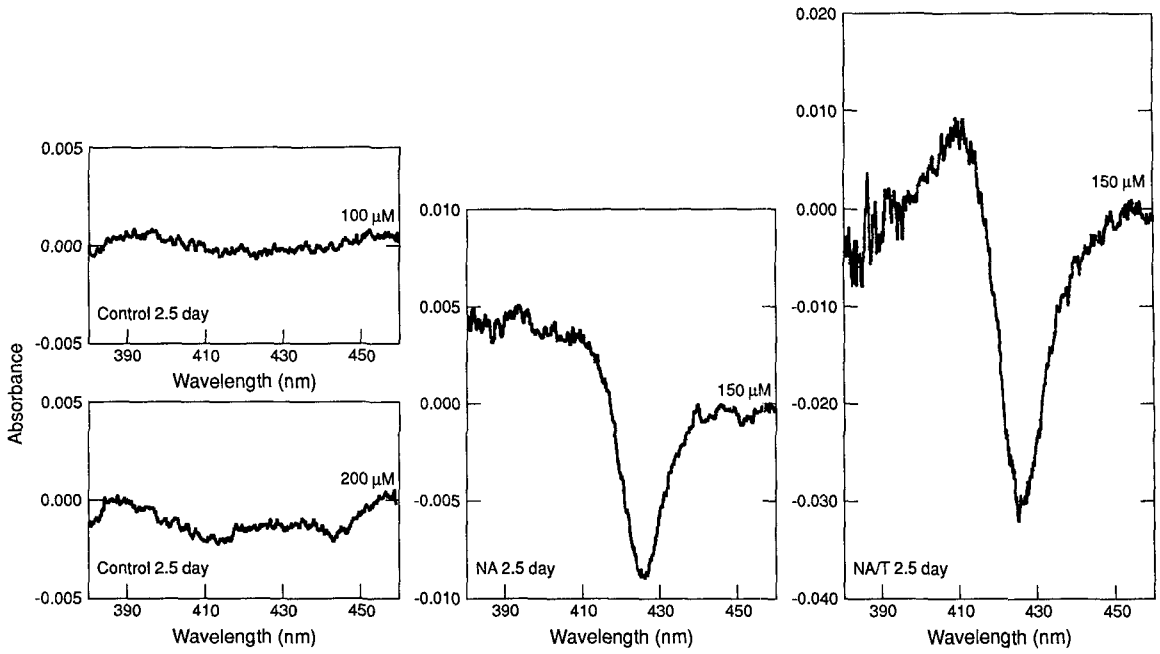

Figure 3. Triasulfuron-binding spectra. Type I-binding spectra were defined using microsomes from control seedlings at 100 or 200 μM triasulfuron (left), 2.5-d-old NA-treated seedlings at 150 μM triasulfuron (middle), and 2.5-d-old NA plus triasulfuron-treated seedlings at 150 μM triasulfuron (right).

coefficients are induced in response to each of these induction regimes. For comparison, wheat triasulfuron hydroxylase has a K_m of 24 μM for triasulfuron (Thalacker et al., 1994) and maize primisulfuron hydroxylase has apparent K_m values of 137 and 47 μM for hydroxylation on the pyrimidine and phenyl rings, respectively (Fonne-Pfister et al., 1990).

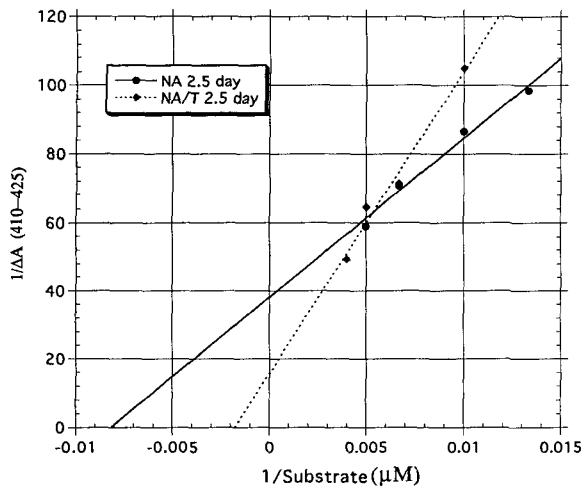

Figure 4. Triasulfuron-binding coefficients. The K_s values for triasulfuron were defined for microsomes isolated from 2.5-d-old NA or NA plus triasulfuron-treated seedlings as described in "Materials and Methods." The P450 content for these microsomes is approximately 40 pmol mg^{-1} protein as estimated by CO difference spectra. The K_s of NA-treated microsomal protein is 123 ± 0.2 μM; the K_s of NA plus triasulfuron-treated microsomal protein is 548 ± 34 μM.

DISCUSSION

These results clearly indicate that the P450 isozyme(s) mediating triasulfuron metabolism in 2.5-d-old maize seedlings is induced significantly in response to NA and only marginally in response to triasulfuron alone. In combination, NA and triasulfuron synergistically induce triasulfuron metabolism to a level at least 88-fold higher than in uninduced seedlings. In 6.5-d-old seedlings, triasulfuron metabolism is induced to the same level by NA and by NA administered in combination with triasulfuron but not by triasulfuron alone. The fact that *t*-CAH activity is not altered by any of these safener/herbicide treatments (Table III) indicates that induction of triasulfuron metabolism is not affected by a general increase in P450 expression but rather by the induction of a specific P450 or a subset of P450s. This conclusion is reiterated in other studies indicating that *t*-CAH activity in wheat and sorghum seedlings is repressed by concentrations of NA (0.5 g NA/100 g seeds) that induce the metabolism of a variety of herbicides (Zimmerlin and Durst, 1992; Zimmerlin et al., 1992; Moreland et al., 1993b).

These metabolic assays also demonstrate that at early stages of germination triasulfuron metabolism is synergistically enhanced by the administration of NA plus triasulfuron. Thereafter, induction occurs only in response to NA. Comparison of the rates at which triasulfuron is metabolized by microsomes obtained from 2.5-d-old NA-treated seedlings versus NA plus triasulfuron-treated seedlings indicates that triasulfuron metabolism is 2.6-fold higher in NA plus triasulfuron-treated seedlings. In 2.5- versus 6.5-d-old NA plus triasulfuron-treated seedlings, triasulfuron metabolism is 2.6-fold higher in the younger seedlings. In

accordance with these data, the triasulfuron-binding spectra show that the triasulfuron-binding P450(s) is absent in control seedlings and 3.2-fold more abundant in 2.5-d-old NA plus triasulfuron-treated seedlings than in NA-treated seedlings. The agreement of the metabolic and triasulfuron-binding activities in the control and induced seedlings indicates that the P450(s) capable of binding and metabolizing triasulfuron accumulates at a high level in response to NA and triasulfuron treatment and at a lower level in response to NA treatment alone. The concomitant reduction in triasulfuron inducibility and binding activity in NA-treated seedlings indicates that this isozyme is induced to a lesser extent and does not simply accumulate in a less active or nonfunctional form. The fact that the NA and NA plus triasulfuron activity levels are identical in 6.5-d-old seedlings suggests that older seedlings have lost their ability to respond to triasulfuron. In agreement with this, triasulfuron by itself is incapable of inducing its own metabolism in 6.5-d-old seedlings; induction occurs in 2.5-d-old seedlings, albeit at a low level compared to that induced by simultaneous treatment with NA. The $MnCl_2$, NA, and triasulfuron induction data (Table II) indicate that triasulfuron insensitivity in older seedlings does not result from a general limitation in the inducibility of this detoxification system: $MnCl_2$ remains capable of stimulating triasulfuron metabolism above that obtained with NA plus triasulfuron. One last suggestion drawn from our analysis of triasulfuron-binding coefficients is that multiple P450s having different binding coefficients are induced in response to NA versus triasulfuron treatment.

The absence of detectable triasulfuron metabolism and binding activity in uninduced seedlings suggests that metabolism of this herbicide in maize is not mediated by an abundant constitutive P450 with an activity directed against one of the endogenous substrates. This contrasts with data suggesting that hydroxylation of the herbicide diclofop in wheat is mediated by the endogenous lauric acid hydroxylase (Zimmerlin and Durst, 1992); in this latter case, diclofop aryl hydroxylase activity exists at a readily detectable level in uninduced seedlings and is induced by NA in parallel with lauric acid hydroxylase activity. It remains possible, therefore, that some herbicides are metabolized by highly specialized P450 isozymes and others are metabolized by endogenous P450s having broader substrate specificities.

The range of naphthalene derivatives that are capable of inducing triasulfuron metabolism is extremely limited. In combination with triasulfuron treatment, the rank order of the derivatives that we have tested as inducers is NA $\gg$ NDA $\gg$ NI = NDiA. The auxin analog NAA only marginally increases triasulfuron metabolism, and naphthalene itself fails to induce metabolism of this herbicide. Although the mode by which these derivatives induce expression of this particular P450 activity is not yet clear, our data definitively demonstrate that induction by NA does not activate developmental and/or tissue-specific pathways that normally respond to auxin or auxin analogs. Nevertheless, NA activation and synergistic enhancement of triasulfuron metabolism in young seedlings depends on the asymmetric positioning of negatively charged moieties on the hydrophobic naphthalene core.

In summary, induction of triasulfuron metabolism can be activated by multiple exogenous chemical cues, which at times act synergistically to enhance metabolism. Our data suggest that at least two induction cascades regulate this metabolic activity. The NA induction cascade operates independently of development; the triasulfuron induction cascade appears to be tightly modulated with respect to the developmental age of the seedlings. The fact that the uptake levels for radioactively labeled sulfonylurea herbicides do not appear to vary in 3- to 7-d-old etiolated maize seedlings (Diehl, 1992) suggests that this second (triasulfuron) induction cascade is limited by a developmentally regulated factor rather than by herbicide uptake. Although we do not presently know how many P450 genes encode this metabolic activity, it appears that multiple isozymes having different binding coefficients are induced in response to NA and triasulfuron. The activation scenario most consistent with our data suggests that multiple, closely related triasulfuron hydroxylase coding sequences exist, with each responding independently to either NA or triasulfuron induction cascades. Activation by multiple chemical and developmental cues might then be expected to exert an additive effect on metabolism.

ACKNOWLEDGMENTS

We gratefully acknowledge CIBA-Geigy for supplying triasulfuron and 5-hydroxytriasulfuron and Drs. D. Moreland, N. Balke, and D.S. Frear for helpful discussions.

Received March 6, 1995; accepted August 21, 1995.

LITERATURE CITED

Bozak KR, O'Keefe DP, Christoffersen RE (1992) Expression of a ripening-related avocado (*Persea americana*) cytochrome P450 in yeast. Plant Physiol **100:** 1976–1981

Bozak KR, Yu H, Sirevag R, Christoffersen RE (1990) Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. Proc Natl Acad Sci USA **87:** 3904–3908

Christopher JT, Powles SB, Liljegren DR, Holtum JAM (1991) Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). Plant Physiol **95:** 1036–1043

Cotterman JC, Saari LL (1992) Rapid metabolic inactivation is the basis for cross-resistance to chlorsulfuron in diclofop-methyl-resistant rigid ryegrass (*Lolium rigidum*) Biotype SR4/84. Pestic Biochem Physiol **43:** 182–192

Diehl KE (1992) Examination of the synergistic interaction between nicosulfuron and terbufos in corn and the inhibition of cytochrome P450 as a mechanism. PhD thesis. University of Illinois, Urbana-Champaign

Donaldson RP, Luster DG (1991) Multiple forms of plant cytochromes P-450. Plant Physiol **96:** 669–674

Durst F, Benveniste I, Salaun J-P, Werck-Reichhart D (1992) Function, mechanism and regulation of cytochrome P-450 enzymes in plants. Biochem Soc Trans **20:** 353–361

Fahrendorf T, Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.). Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. Arch Biochem Biophys **305:** 509–515

Fonne-Pfister R, Gaudin J, Kreuz K, Ramsteiner K, Ebert E (1990) Hydroxylation of primisulfuron by an inducible cytochrome P450-dependent monooxygenase system from maize. Pestic Biochem Physiol **37:** 165–173

Fonne-Pfister R, Kreuz K (1990) Ring-methyl hydroxylation of chlortoluron by an inducible cytochrome P450-dependent enzyme from maize. Phytochemistry **29:** 2793–2796

Frear DS, Swanson HR, Thalacker FW (1991) Induced microsomal oxidation of diclofop, triasulfuron, chlorsulfuron, and linuron in wheat. Pestic Biochem Physiol **41:** 274–287

Funk C, Croteau R (1993) Induction and characterization of a cytochrome P-450-dependent camphor hydroxylase in tissue cultures of common sage (*Salvia officinalis*). Plant Physiol **101:** 1231–1237

Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, Menting JGT, Lu C-Y, Farcy E, Stevenson TW, Cornish EC (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. Nature **366:** 276–279

Jefcoate CR (1978) Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. Methods Enzymol **52:** 258–279

Lamoureux GL, Rusness DG (1986) Xenobiotic conjugation in higher plants. *In* GD Paulson, J Caldwell, DH Hutson, JJ Menn, eds, Xenobiotic Conjugation Chemistry, Symposium Series 299. American Chemical Society, Washington, DC, pp 62–105

McFadden JJ, Frear DS, Mansager ER (1989) Aryl hydroxylation of diclofop by a cytochrome P450 dependent monooxygenase from wheat. Pestic Biochem Physiol **34:** 92–100

Mizutani M, Ward E, DiMaio J, Ohta D, Ryals J, Sato R (1993) Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 ($P450_{C4H}$) possessing cinnamate 4-hydroxylase activity. Biochem Biophys Res Commun **190:** 875–880

Moreland DE, Corbin FT, McFarland JE (1993a) Oxidation of multiple substrates by corn shoot mirocomes. Pestic Biochem Physiol **47:** 206–214

Moreland DE, Corbin FT, McFarland JE (1993b) Effects of safeners on the oxidation of multiple substrates by grain sorghum microsomes. Pestic Biochem Physiol **45:** 43–53

Mougin C, Cabanne F, Canivenc M-C, Scalla R (1990) Hydroxylation and *N*-demethylation of chlortoluron by wheat microsomal enzymes. Plant Sci **66:** 195–203

Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem **239:** 2370–2378

Reichhart D, Salaun J-P, Benveniste I, Durst F (1980) Time course of induction of cytochrome P450, NADPH-cytochrome c reductase, and cinnamic acid hydroxylase by phenobarbital, ethanol, herbicides, and manganese in higher plant microsomes. Plant Physiol **66:** 600–604

Teutsch HG, Hasenfrantz MP, Lesot A, Stoltz C, Garnier J-M, Jeltsch J-M, Durst F, Werck-Reichhart D (1993) Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proc Natl Acad Sci USA **90:** 4102–4106

Thalacker FW, Swanson HR, Frear DS (1994) Characterization, purification, and reconstitution of an inducible cytochrome P450-dependent triasulfuron hydroxylase from wheat. Pestic Biochem Physiol **49:** 209–223

Urban P, Werck-Reichhart D, Teutsch HG, Durst F, Regnier S, Kazmaier M, Pompon D (1994) Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Eur J Biochem **222:** 843–850

Vetter H-P, Mangold U, Schroder G, Marner F-J, Werck-Reichhart D, Schroder J (1992) Molecular analysis and heterologous expression of an inducible cytochrome P-450 protein from periwinkle (*Catharanthus roseus* L.). Plant Physiol **100:** 998–1007

Werck-Reichhart D, Batard Y, Kochs G, Lesot A, Durst F (1993) Monospecific polyclonal antibodies directed against purified cinnamate 4-hydroxylase from *Helianthus tuberosus*. Plant Physiol **102:** 1291–1298

Zimmerlin A, Durst F (1990) Xenobiotic metabolism in plants: aryl hydroxylation of diclofop by a cytochrome P-450 enzyme from wheat. Phytochemistry **29:** 1729–1732

Zimmerlin A, Durst F (1992) Aryl hydroxylation of the herbicide diclofop by a wheat cytochrome P-450 monooxygenase. Plant Physiol **100:** 874–881

Zimmerlin A, Salaun 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