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Monooxygenases from Soybean Root Nodules: Aldrin Epoxidase and Cinnamic Acid 4-Hydroxylase

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The metabolism of aldrin and *trans*-cinnamic acid within the root nodules of soybean (*Glycine max.* cv. Forrest) was investigated. *In vitro* studies with microsomal preparations revealed the presence of two distinct monooxygenase enzymes requiring NADPH and molecular oxygen. This was shown by different distributions in membrane fractions from sucrose density gradients. In addition, cinnamic acid hydroxylase was sensitive to carbon monoxide (CO), similar to cytochrome *P*-450-dependent monooxygenases, whereas aldrin epoxidation was not. Cyanide ion, strongly inhibited the epoxidase, without affecting hydroxylase activity. The superoxide radical (O_2^-) scavengers, superoxide dismutase and norepinephrine, inhibited aldrin epoxidation but allowed greater cinnamic acid 4-hydroxylase activity. These results indicate aldrin epoxidase, in contrast to cinnamic acid hydroxylase, does not involve cytochrome *P*-450, and may instead utilize a peroxidase-like hemoprotein.

INTRODUCTION

Biological oxidations of pesticides, drugs, amino acids, and hormones by microsomal monooxygenase (mixed-function oxidase) enzymes in animals, insects, and plants have been reviewed (1–3). From those studies it is now clear that in animals and insects a hemoprotein called cytochrome *P*-450 is the terminal component of the electron transport chain for the detoxication of foreign compounds. However, with plants there are uncertainties about the nature and mechanism of such mixed-function oxidase enzymes. Several of these plants appear to be cytochrome *P*-450 dependent, similar to those of animals (4, 5), whereas other plant oxygenases seem to be different (6), perhaps involving peroxidases.

In an earlier report (7) we described the occurrence of a monooxygenase system within the root nodules of the nitrogen-fixing legume, soybean. Particulate fractions from the nodules of soybean epoxidatively attacked the chlorinated insecticide aldrin when assayed by *in vitro* methods. This reaction required NADPH and molecular oxygen, similar to animal mixed-function oxidase systems. However, particularly in crude fractions, NADPH also catalyzed an inactivation of aldrin epoxidase, apparently as a result of an interaction with other low-molecular-weight substances. We now report experiments which demonstrate the occurrence of two distinct monooxygenase enzyme systems in soybean nodules.

MATERIALS AND METHODS

Chemicals. [3^{14}C]Cinnamic acid (sp act 58 mCi/mmol) was obtained from Amersham, Australia. Aldrin (99.9% pure) and dieldrin (99.7% pure) were kindly provided by Shell Chemical (Australia) Pty Ltd. Unlabeled *trans*-cinnamic acid, *p*-coumaric acid, superoxide dismutase, (±)-arterenol (norepinephrine), and $NADP^+$ were from Sigma; glucose 6-P from Calbiochem-Behring, and glucose 6-P dehydrogenase was purchased from Boehringer Mannheim. TLC aluminium roll silica gel 60 F_{254} was obtained from E. Merck.

Plant Material. Soybean (*Glycine max:* cv forrest 78-6008) seeds were surface ster-

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ilized with 70% ethyl alcohol for 5 min and then washed several times first under tap and then distilled water. Seeds were inoculated with *Rhizobium japonicum* CB 1809, and planted in Perlite medium and provided with N-free nutrient solution (8), while growing in a greenhouse. The plants were removed at 4 weeks and the roots washed under running tap water. Nodules were detached by hand and used for epoxidase and hydroxylase preparations.

Membranous extracts were prepared by homogenizing nodules in a deoxygenated sucrose (0.3 *M*) K-phosphate (0.1 *M*) buffer, pH 7 (5 ml/g fresh weight) with 2% (w/v) polyclar AT (insoluble polyvinylpyrrolidone) under nitrogen in a glass mortar kept below 4°C. The resulting slurry was squeezed through cheesecloth and centrifuged at 1000*g* for 20 min, producing a crude brei as supernatant. The brei was then centrifuged at 12,000*g* for 20 min, and the pellet discarded. The clear supernatant was further centrifuged at 105,000*g* for 60 min, in a Beckman L2-65 ultracentrifuge, using a Ti-50 rotor. The pelleted membranes (including membranes of smooth and rough microsomes from endoplasmic reticulum, plasma membranes, and peribacteroid membranes) were resuspended in the sucrose phosphate buffer using a Potter–Elvehjem homogenizer fitted with a Teflon pestle.

Aldrin epoxidase assays were carried out with the membranous material following the method used for crude homogenates (7). Analyses for dieldrin were performed by electron capture gas chromatography (7). The standard deviation of repeated assays was 6.5% of the mean values. Each reaction mixture contained about 0.03 mg protein. Cinnamic acid hydroxylase was assayed by a procedure modified from Reichhart *et al.* (9). In a final volume of 200 μl, the reaction mixture contained 0.35 μmols $NADP^+$, 3.5 μmols glucose 6-P, 0.2 units glucose 6-P dehydrogenase, 0.9 nmols *trans*-[3-^{14}C]cinnamic acid (58 mCi/mmol), and 67.5 nmols (10 μg) unlabeled *trans*-cinnamic acid. Protein contents were similar to that of the aldrin epoxidase assay. The reaction mixture was incubated in a shaking water bath at 30°C for 30 min, after which the reaction was stopped with 25 μl of 5 *M* HCl. Carrier *trans*-cinnamic acid and *p*-coumaric acids (0.1 mg in 10 μl ethanol) were added and precipitated protein removed by centrifugation. The clear aqueous solution (50–100 μl) was spotted on DC Alurolle Kieselgel 60 F254 aluminium thin-layer strips and developed over toluene/acetic acid/water (6:7:3) mixture by ascending chromatography. Chromatogram spots were identified under uv light (R_f value for cinnamic acid, 0.41 and coumaric acid, 0.13) and the coumaric acid spots cut out and counted in a Packard Minaxi Tri-carb 4000 liquid scintillation counter using Bray's aqueous scintillant (10).

Sucrose density gradients (20–50% w/w) were prepared as reported elsewhere (7). Crude brei (2 ml) was layered on the top of the gradient and spun at 25,000 rpm for 150 min in a Beckman L2-65 ultracentrifuge, using a swing-out rotor (SW27). Gradient fractions were collected (3 ml) by upward displacement using a colored (phenol red) sucrose solution (60% w/w). The density of each fraction was calculated by measuring the refractive index using a refractometer.

Membranous protein content was measured at 595 nm using the Coomassie brilliant blue binding method (11).

RESULTS AND DISCUSSION

Influence of Inhibitors on Aldrin Epoxidation and Cinnamic Acid Hydroxylation

The action of various inhibitors and cofactors was tested on both aldrin epoxidation and cinnamic acid hydroxylation with an added NADPH-generating system. The data (Table 1) show that aldrin epoxidase and cinnamic acid hydroxylase behave differently with different compounds. Thus aldrin epoxidation was strongly inhibited

TABLE 1

Effect of Cofactors and Inhibitors on Aldrin Epoxidase and Cinnamic Acid 4 Hydroxylation Activity of Soybean Nodule Membranous Material (100,000 g pellet)

Incubation mixture		Aldrin epoxidase % of control	Cinnamic acid 4 hydroxylation % of control
Control (+NADPH)		100	100
− NADPH		79.0	61.0
KCN	(1 m*M*)	2.3	92.5
EDTA	(1 m*M*)	8.2	64.5
Mercaptoethanol	(3 m*M*)	0.9	108.0
Benzoquinone	(1 m*M*)	7.7	14.4
Hydroquinone	(1 m*M*)	12.3	36.0
Triton X-100	(0.05% w/v)	86.4	32.2
Sodium azide	(1 m*M*)	90.9	25.1
Cinnamic acid	(1 m*M*)	109.0	—
Ethanol	(0.1 *M*)	90.0	100.7
Antimycin A	(0.05 m*M*)	19.6	50.5
Catalase	(100 units)	64.8	100.0
2:2 Dipyridyl	(1 m*M*)	80.8	86.5
Cyt.*C*	(0.1 m*M*)	18.2	37.4
Superoxide dismutase (SOD)	(100 units)	74.4	108.0
SOD	(300 units)	53.3	115.5
Norepinephrine	(0.1 m*M*)	47.2	125.4
CO (dark)	(10%)	91.0	57.2

Note. Membranous material prepared as described under Materials and Methods and enzymic assays were carried out with added NADPH generating system. Protein about 0.03 mg/assay. Results are the mean of two replicated experiments.

by 1 m*M* KCN while cinnamic acid hydroxylation was unaffected. Even at 0.1 m*M* KCN, 90% inhibition was observed with aldrin epoxidation (result not shown). Such a result was expected for a peroxidase type monooxygenase reaction, as with the case of pea root epoxidation (12) and animal myeloperoxidase enzymes (13, 14). Cinnamic acid hydroxylase is reported as being cyanide insensitive (4, 15) agreeing with our finding. Further confirmation of the different action of KCN with two substrates is shown later in Fig. 2.

The metal chelating agent, EDTA, inhibited aldrin epoxidation 50% at 0.01 m*M*. At 1 m*M*, even greater inhibition (90%) was observed and efforts to remove the inhibition with excess Ca^{2+} and Mg^{2+} ions (5 m*M*) showed very little effect. This suggests the formation of an EDTA metal complex more stable than the Ca^{2+} or Mg^{2+} ion complexes, or some other effect. EDTA, however, showed only about 40% inhibition at 1 m*M* with cinnamic acid hydroxylation.

The addition of mercaptoethanol stimulated cinnamic acid hydroxylase as expected (4); however, we did not include mercaptoethanol in our preparations as it strongly inhibited aldrin epoxidation. This facilitated the comparative study of aldrin epoxidase and cinnamic acid hydroxylase. Quinones were generally inhibitory to both aldrin epoxidase and cinnamic acid hydroxylase activities. Azide, which inhibits animal peroxidases, did not significantly affect the nodule epoxidase system. Klebaroff and Rosen (14) reported that the effect of azide varied with the substrate employed. They found stimulation of ethylene production with methional oxidation in the presence of azide, which they suspected was a myeloperoxidase independent activity. The ionic detergent Triton X-100,

caused loss of activity with both aldrin epoxidation and cinnamic acid hydroxylation, but the effect was greater with the latter. Variable loss of activity in cinnamic acid hydroxylation with Triton X-100 has been reported earlier (16).

Scavengers of the superoxide free radical such as superoxide dismutase and norepinephrine inhibited aldrin epoxidation, but stimulated cinnamic acid hydroxylation. This suggests the involvement of free radicals in the aldrin epoxidase reaction, also differentiating it from cinnamic acid hydroxylase. Supporting evidence for two different systems is the effect of carbon monoxide, which inhibited cinnamic acid hydroxylation. Since the carbon monoxide effect is light reversible (4) experiments with CO were carried out in the absence of light. The inhibitory action of carbon monoxide on cinnamic acid hydroxylation agrees with other reports (5, 9, 15, 17, 18) as cinnamic acid hydroxylase activity is a cytochrome *P*-450-dependent hydroxylation.

Time Courses of Cinnamic Acid Hydroxylase and Aldrin Epoxidase

The time course of cinnamic acid hydroxylation by a 12,000–105,000*g* membrane fraction, obtained by differential centrifugation, showed a rate of activity declining with time with ± NADPH, reaching a maximum production in 1 hr. Thereafter *p*-coumaric acid (4-hydroxy cinnamic acid) accumulation ceased, presumably due to substantial inactivation of the hydroxylase. A slow disappearance of *p*-coumaric acid after 1 hr incubation could have been due to further hydroxylation of *p*-coumaric to caffeic (3-4 dihydroxy cinnamic) acid or a related compound. The hydroxylation of *p*-coumaric to caffeic acid by the chloroplast of spinach beet has been reported (19). No attempts were made to identify such compounds in our system.

It was surprising to note that even cinnamic acid hydroxylase had substantial endogenous activity in the absence of an NADPH generating system, similar to that of aldrin epoxidation (6, 7). Data obtained with aldrin epoxidase (Table 2) showed that the endogenous activity was a persistent effect, that could be reduced only to a small extent by washing of membranes. Repeated washing by resuspension of

TABLE 2

Effect of NADPH on Aldrin Epoxidase in Centrifugal Fractions Isolated from Soybean Root Nodules

Fraction	Dieldrin (nmol/mg protein)	
	− NADPH	+ NADPH
Crude brei (300*g* SN)	0.50	0.75
12,000*g* SN	0.35	0.36
12,000*g* P	2.71	2.38
105,000*g* P	10.90	7.77
105,000*g* P (washed, once)	13.68	5.28
105,000*g* P (washed, twice)	5.32	2.87
105,000*g* SN	0.18	0.18

Note. Material prepared as described under Materials and Methods. Reaction mixture contained ±NADPH generating system and 5 μg aldrin per assay. Incubation was for 15 min, with extraction and detection as described under Materials and Methods. Results shown are the mean of replicated experiment with standard deviation of not more than 6.5% of the value. SN: Supernatant, P: pellet.

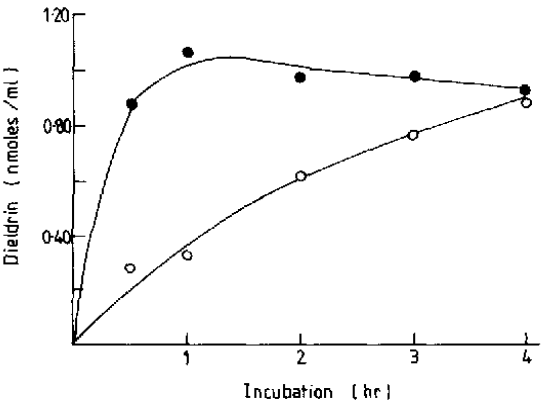

FIG. 1. *Time course of aldrin epoxidation with membranous material from a sucrose density gradient. Linear sucrose density gradients were prepared as described under Materials and Methods and the material of density 1.16 was used. Aldrin concentration was 5 μg/0.03 mg proteinlassay. Activity with* (●) *and without* (○) *NADPH are shown. Each point represents the average of duplicated experiments with standard deviation of less than 6% of the value.*

pellets resulted in loss of activity to the same extent for both ± NADPH. It can be noted that sucrose density gradient fractionation should be an excellent washing technique, but even there, the NADPH requirement for aldrin epoxidase was not absolute (7). At this stage the identity of the endogenous electron donors is not clear, but presumably they are lipophilic.

For time courses of aldrin epoxidase (12,000–105,000*g* fraction), addition of an NADPH generating system was stimulatory only in incubations of less than 1 hr. By then the rate of activity had almost declined to zero, presumably as a result of an NADPH catalyzed inactivation of aldrin epoxidase. Without added NADPH, continuing production of dieldrin using endogenous reductant was obtained for several hours, though at a declining rate. We earlier reported the inactivation of aldrin epoxidase with added NADPH in soybean nodule crude homogenates (7). Here, we have studied this inactivation of the epoxidase enzyme using the material obtained by linear sucrose density gradients. Active membranous material (ρ 1.16) was assayed for time course and the results shown in Fig. 1. Activity with added NADPH was much greater than that of the membranous material obtained by differential centrifugation. However, epoxidase activity declined to zero after 1 hr incubation, similar to the previous experiments above. Here, again, dieldrin production without NADPH continued and reached a similar total amount after 4 hr incubation.

The nature of the NADPH-catalyzed inactivation of both enzymes needs further study, but the observation that cinnamic acid hydroxylase was more active with the superoxide scavengers, SOD and norepinephrine (Table 1), suggests that free radical attack may be involved. However, if superoxide formation is part of the mechanism of aldrin epoxidase, as we suggest, such scavengers would here be inhibitory, as was observed (Table 1). To test such ef-

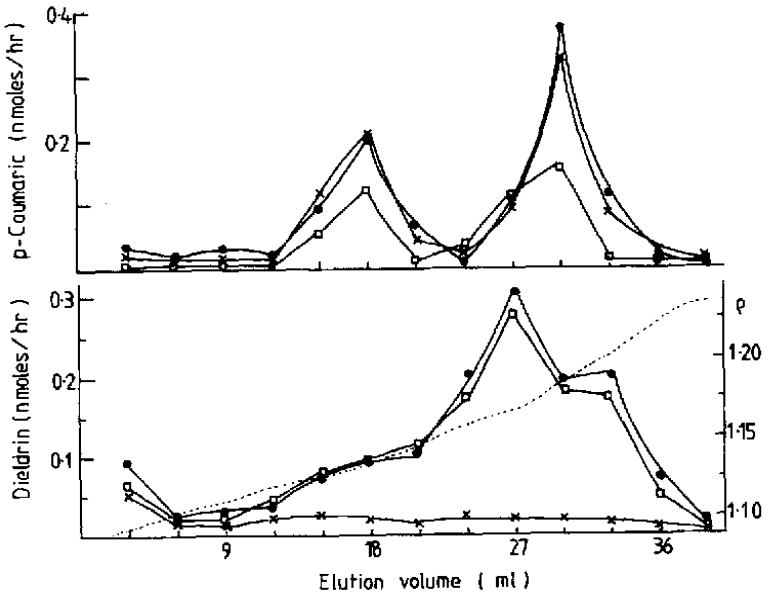

FIG. 2. *Effect of carbon monoxide and cyanide ion with sucrose density gradient fractions on cinnamic acid hydroxylase and aldrin epoxidase activity. Brei (2 ml) equivalent to 1 g of nodule fresh weight was layered on a sucrose gradient (20–50%, w/w) prepared as described under Materials and Methods. The distribution of hydroxylase [(●) + NADPH], epoxidase [(●) + NADPH], and the effect of carbon monoxide 10% (□), KCN 1 mM (X) and density (– – – –) of 3-ml fractions are shown. Values shown represent the means of two experiments.*

fects on both enzymes, it was necessary to restrict reactions to 30 min or less, before serious inactivation had occurred (Tables 1, 2).

Membrane Localization of Monooxygenases

The location of aldrin epoxidase and cinnamic acid hydroxylase activities within the nodules was investigated by linear sucrose density gradient techniques. The results are shown in Fig. 2. In the case of aldrin epoxidation density gradient fractions indicate the active sites to be localized in tubes with densities (ρ) of 1.08, 1.13, and 1.16, respectively. However, with cinnamic acid hydroxylation, activity was principally associated with both ρ = 1.13 and ρ = 1.19 regions. Similar sites of activities were reported for castor bean endosperm hydroxylase (17) showing significant activities at ρ = 1.12, ρ = 1.19 and 1.22. In the case of castor bean with use of marker enzymes, the principal acitivity was associated with the microsomes of endoplasmic reticulum (ρ 1.12) and the residual activities were thought to be associated with the mitochondria (ρ 1.19) and peroxisomes (microbodies), ρ 1.22. It is clear from Fig. 2 that the sites of activity are different for both aldrin epoxidase and cinnamic acid hydroxylase. Also shown in Fig. 2 are the differential effects of CO and KCN inhibition.

The role of cinnamic acid hydroxylase in plants is clear (21), but such is not the case with aldrin epoxidase. Further studies are in progress to examine the significance and detailed mechanism of aldrin epoxidase in plant roots and soybean nodules.

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