Kinetic studies on protoporphyrinogen oxidase inhibition by diphenyl ether herbicides

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Diphenyl ethers (DPEs) and related herbicides are powerful inhibitors of protoporphyrinogen oxidase, an enzyme involved in the biosynthesis of haems and chlorophylls. The inhibition kinetics of protoporphyrinogen oxidase of various origins by four DPEs, (methyl)-5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid (acifluorfen and its methyl ester, acifluorfen-methyl), methyl-5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-chlorobenzoate (LS 820340) and methyl-5-[2-chloro-5-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid (RH 5348), were studied. The inhibitions of the enzymes from maize (*Zea mays*) mitochondrial and etiochloroplastic membranes and mouse liver mitochondrial membranes were competitive with respect to the substrate, protoporphyrinogen IX, for all four molecules. The relative efficiencies of the inhibitors were: acifluorfen-methyl > LS 820340 > RH 5348 > acifluorfen. The four molecules showed mixed-competitive type inhibition of the enzyme from yeast mitochondria where acifluorfen, a carboxylic acid, had the same inhibitory activity as its methyl ester, acifluorfen-methyl, and both were much greater than that of LS 820340 and RH 5348.

INTRODUCTION

Diphenyl ether (DPE) herbicides are peculiar in that they kill plants only in the light; they induce peroxidative degradation of cellular constituents, especially membrane lipids (Orr & Hess, 1982), which leads to cell lysis.

Plants treated with DPEs accumulate high levels of a tetrapyrrole pigment, protoporphyrin IX (Matringe & Scalla, 1987; Lydon & Duke, 1988; Matringe & Scalla, 1988b; Sandmann & Böger, 1988; Witkowski & Halling, 1988), indicating that the DPEs interfere with the haem- and/or chlorophyll-biosynthetic pathways. Porphyrins can generate singlet oxygen in the light (Hopf & Whitten, 1978). Thus their accumulation in treated plants explains the light-dependent peroxidations of membrane lipids and the ensuing membrane disruptions evoked by DPEs, providing a plausible mechanism for the primary mode of action of DPEs (Matringe & Scalla, 1987, 1988b).

We have demonstrated that DPE herbicides are powerful inhibitors of protoporphyrinogen oxidase, the last enzyme of the common branch of the haem- and chlorophyll-biosynthetic pathways in plants (Matringe *et al.*, 1989a). This finding was confirmed (Witkowski & Halling, 1989). Although it appears that all the initial steps of haem and chlorophyll biosynthesis take place within the plant chloroplast (for a review see Beale & Weinstein, 1990), protoporphyrinogen oxidase is present in both the mitochondrial and chloroplast membranes (Jacobs & Jacobs, 1987), and both activities are inhibited by DPEs (Matringe *et al.*, 1989a).

DPEs do not inhibit only protoporphyrinogen oxidase of plant origin: mouse and yeast (*Saccharomyces cerevisiae*) mitochondrial enzymes are equally sensitive to these herbicides (Matringe *et al.*, 1989a). Furthermore, three other chemically unrelated peroxidizing herbicidal molecules, 5-*t*-butyl-3-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2-one

(oxadiazon), the pyridine derivative (S)-*N*-(methylbenzyl)-carbamoyl-5-propionyl-2,6-lutidine (LS 82556) and the pyrazole derivative 5-amino-4-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)pyrazol (M&B 39279) also produce comparable protoporphyrin IX accumulation *in vivo* (Duke *et al.*, 1989; Matringe & Scalla, 1988a) and protoporphyrinogen oxidase inhibition *in vitro* (Matringe *et al.*, 1989b).

Thus protoporphyrinogen oxidase appears to be the primary target for a number of herbicidal molecules, and an analysis of kinetics and mechanism of the interaction of the enzyme with its inhibitors should provide information on the mode of action of these herbicides. We have therefore investigated the mechanism of protoporphyrinogen oxidase inhibition by four related DPE molecules (Fig. 1). The molecules are (methyl)-5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid [acifluorfen (AF) and its methyl ester acifluorfen-methyl (AFM)], two typical nitrodiphenyl ether herbicides, and methyl-5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-chlorobenzoate [LS 820340 (LS)], which differ from AFM in having a chlorine atom instead of a nitro substituent, but nevertheless exerts the same type of light-dependent herbicidal activity (Ensminger & Hess, 1985), and methyl-5-[2-chloro-5-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid [RH 5348 (RH)], in which the displacement of a CF₃ group results in a lowered phytotoxicity (Becerril & Duke, 1989).

MATERIALS AND METHODS

Chemicals

AF (sodium salt) was from ChemService, West Chester, PA, U.S.A.; AFM and LS 820340 were provided by Rhône-Poulenc Agrochimie, Lyon, France. RH 5348 was a gift from Rohm and Hass, Philadelphia, PA, U.S.A. Protoporphyrin IX hydrochloride was from Porphyrin Products, Logan, UT, U.S.A.

Abbreviations used: AF(M), acifluorfen(-methyl), (methyl)-5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid; LS, LS 820340 {methyl-5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-chlorobenzoate}; RH, RH 5348 {methyl-5-[2-chloro-5-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid}; DTT, dithiothreitol; DPEs, diphenyl ethers; oxadiazon, 5-*t*-butyl-3-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2-one; LS 82556, (S)-*N*-(methylbenzyl)carbamoyl-5-propionyl-2,6-lutidine; M&B 39279, 5-amino-4-cyano-1-(2,6-dichloro-4-trifluoromethylphenyl)pyrazol; IC₅₀ is defined in the text.

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Fig. 1. Chemical structures of AF, AFM, LS 820340 and RH 5348

Preparation of biological samples

Etiolated maize (*Zea mays*) seedlings were grown as described by Clément *et al.* (1986). Maize seeds (cv. 'Monclair') were surface-sterilized with calcium hypochlorite, soaked in water for 1 day and germinated on a stainless-steel screen above distilled water for 6–7 days in darkness. Just before etiochloroplast isolation, seedlings were allowed to green for 2–4 h in dim light ($30 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ photosynthetically active radiation).

Eti chloroplasts were isolated by the procedure of Pardo *et al.* (1980). Maize mitochondria were prepared as described by Jackson & Moore (1979). Contamination of the mitochondrial fractions by etiochloroplastic membranes was evaluated by measuring the amount of carotenoids and chlorophylls extracted by 80 % (v/v) acetone; the reciprocal contamination was assessed by measuring cytochrome *c* oxidase activity in the etiochloroplastic fractions. According to these criteria, cross-contaminations were $\leq 10\%$.

Yeast (*Saccharomyces cerevisiae*) mitochondrial membranes were prepared from the haploid laboratory strain FL 200 grown and processed as described previously (Urban-Grimal & Labbe-Bois, 1981).

Mouse (Swiss strain) liver mitochondria were isolated by standard procedures (Johnson & Lardy, 1967).

Assay of protoporphyrinogen oxidase activity

Protoporphyrinogen IX oxidase activity was assayed spectrofluorimetrically at 30°C as described by Labbe *et al.* (1985), by measuring the initial velocity of protoporphyrin IX formation from protoporphyrinogen prepared by chemical reduction (Jacobs & Jacobs, 1982) of protoporphyrin IX by 3% sodium amalgam in the presence of 50 mM-ascorbic acid. The excitation and emission wavelengths were 410 nm and 633 nm respectively. The standard reaction medium (1 ml) contained: 0.1 mmol of potassium phosphate buffer saturated with air (final pH 7.2), 1 μmol of EDTA, 5 μmol of DTT, 0.3 mg of Tween 80, protoporphyrinogen, and 0.5–1 mg of protein. The protoporphyrinogen IX concentration varied from 0.05 μM to 0.5 μM . All inhibitors were dissolved in anhydrous dimethyl sulphoxide; the final solvent concentration was 1% (v/v) in all assays, including controls. The concentration of dissolved oxygen (the other substrate for the enzyme) was always saturating.

Under our assay conditions, the maize protoporphyrinogen oxidases had a different pH optimum (pH 7.2) than that reported

for the barley (*Hordeum vulgare*) enzymes (pH 6.5) (Jacobs & Jacobs, 1987); accordingly all our experiments were done at pH 7.2.

Estimation of protein concentration

Protein concentrations were determined by the method of Bradford (1976), with BSA as a standard.

Statistics

All data represent the mean values of at least three replicates where the variations were $< 5\%$ (10% for maize etioplastic fractions). Statistical calculations were performed with a dedicated software (Statworks; Cricket Corporation, Malverne, PA, U.S.A.).

RESULTS

Determination of the conditions of inhibition assays

The maximum velocities and Michaelis constants of the four enzymes assayed in the absence of inhibitor are summarized in Table 1.

In order to estimate useful concentrations of inhibitors for the determination of the kinetic constants we measured the IC_{50} for AF, AFM, LS and RH on the different sources of enzymes (Table 1) (IC_{50} is the inhibitor concentration producing 50% inhibition under initial-velocity conditions with saturating substrate concentration).

Inhibitory constants for inhibition of protoporphyrinogen oxidase activities by DPEs

The inhibitory effects of the DPE molecules were evaluated by measuring the apparent affinity of each membrane-bound enzyme for its tetrapyrrole substrate, i.e. protoporphyrinogen IX, at various inhibitor concentrations.

AFM affected the K_m for protoporphyrinogen, but not the V_{max} , of the enzymes from maize etiochloroplasts, maize mitochondria and mouse mitochondria (Fig. 2). Thus AFM inhibition was competitive. AFM altered both the K_m for protoporphyrinogen and the V_{max} of the yeast enzyme activity, suggesting that inhibition was mixed-competitive in this case.

The three other DPEs tested acted similarly. The inhibition parameters were determined by graphical analysis of the

Table 1. Maximum velocities ($V_{\max}$) and Michaelis constants (K_m) for protoporphyrinogen IX and experimental IC_{50} values for AF, AFM, LS and RH of protoporphyrinogen oxidase from maize mitochondria (Mito.) and etiochloroplasts (Etio.), mouse liver and yeast mitochondria

Parameter (units)	Enzyme origin ... Organelle ...	Maize		Mouse	Yeast
		Mito.	Etio.	Mito.	Mito.
$V_{\max}$ (nmol $\cdot$ h $^{-1}$ $\cdot$ mg $^{-1}$)		5.5	6.5	7.4	7.2
K_m (μ M)		0.42	0.38	0.24	0.12
IC_{50} (nM)					
AF		500	500	250	9
AFM		6	4	19.5	7
LS		60	10	100	20
RH		700	180	3250	2000

Fig. 2. Lineweaver-Burk plots

Plots of protoporphyrinogen oxidase activity versus protoporphyrinogen IX concentration at various concentrations of AFM for maize mitochondria (a), maize etiochloroplasts (b), Swiss-mice mitochondria (c) and yeast mitochondrial membranes (d) are shown. Values against curves are AFM concentrations in nM.

Table 2. Inhibition constants (K_i) of protoporphyrinogen oxidase activities by DPEs

The K'_i value is also given (in parentheses) for the yeast enzyme, since the inhibition is mixed competitive in this case. Abbreviations: Mito, mitochondria; Etio, etiochloroplasts.

Inhibitor	Origin ... Organelle ...	K_i value (nM)			
		Maize		Mouse	Yeast
		Mito.	Etio.	Mito.	Mito.
AF		80	120	40	7 (63)
AFM		0.8	0.7	3.2	11 (67)
LS		4	2	12	25 (43)
RH		50	30	600	660 (1800)

Table 3. Experimental IC_{50} values for mitochondrial protoporphyrinogen oxidase preparations

Inhibitor	Origin ... Species ...	IC_{50} (nM)				
		Plant		Mouse		
		Potato	Maize	Strain ...	DBA/2	Swiss
AF		50	500		20	250
AFM		0.4	6		3.2	19.5
LS		3	60		16	100
RH		10	700		250	3250

Lineweaver-Burk plots of protoporphyrinogen oxidase activities (Dixon & Webb, 1979). The K_i values were determined from secondary plots of the slopes calculated from the primary plots (Lineweaver-Burk plots) versus inhibitor concentrations. The K'_i for the yeast enzyme was determined from secondary plot of the intercepts on $1/v$ axis (from Lineweaver-Burk plots) versus inhibitor concentrations. All the secondary plots were linear.

Table 2 summarizes the values of the inhibition constants (K_i and K'_i) of the various molecules and enzymes.

The two kinetic parameters of competitive inhibition (but not of other modes of inhibition), IC_{50} and K_i , are related as follows:

$$K_i = IC_{50} / [(s/K_m) - 1]$$

The determination of both K_m and K_i allowed us to verify our data by comparing experimental IC_{50} values with those calculated using above equation. Table 2 shows that there was a good correlation ($r = 0.987$) between these two sets of values, except for the yeast mitochondrial enzyme, where the inhibitions were not purely competitive (results not shown).

Comparisons of the present and previously reported (Matringe *et al.*, 1989a) data showed puzzling differences in reactivity of the mitochondrial protoporphyrinogen oxidases from two plant species [maize as against potato (*Solanum tuberosum*) tuber] or mouse strains (Swiss as against DBA/2). We therefore determined the IC_{50} for AF with DBA/2 and potato mitochondria, since this compound was omitted from our previous study. The IC_{50} values for AFM, LS and RH were confirmed on the same materials and found to be strictly identical with those previously measured. The comparative results are summarized in Table 3.

In order to determine whether the differences in reactivity toward DPEs were due to some differences in the catalytic properties of protoporphyrinogen oxidase without inhibitor, we measured the apparent Michaelis constants for protoporphyrinogen of DBA/2 liver and potato mitochondrial protoporphyrinogen oxidases. The K_m for protoporphyrin-

Table 4. Correlation between experimental IC_{50} values (E) and IC_{50} calculated (C) from the K_i data

Abbreviations: Mito., mitochondria; Etio., etioplasts.

Inhibitor	IC_{50} (nM)					
	Maize				Mouse	
	Mito.		Etio.		Mito.	
	E	C	E	C	E	C
AF	500	680	500	510	250	290
AFM	6	7	4	3	19.5	24
LS	60	35	10	8.5	100	88
RH	700	425	180	130	3250	4400

ogen was identical in DBA/2 and in Swiss-mouse mitochondria ($0.24 \pm 0.02 \mu\text{M}$), but was lower in potato mitochondria ($0.09 \pm 0.01 \mu\text{M}$) than in maize mitochondria ($0.42 \pm 0.02 \mu\text{M}$).

DISCUSSION

The kinetics of inhibition of plant, mouse and yeast protoporphyrinogen oxidase activities by four related DPE herbicides were determined. Membrane-bound enzyme preparations were used which gave apparent kinetic constants and provided new information on the relationships between the enzymes and inhibitors assayed.

DPEs competitively inhibit protoporphyrinogen oxidase from mouse liver mitochondria and maize mitochondrial and etiochloroplastic membranes and are mixed inhibitors of the yeast enzyme. A linear relationship between the IC_{50} and K_i applied for the mouse and plant protoporphyrinogen oxidases, but not for the yeast enzyme, confirming the differences in the mode of inhibition between these enzymes.

Studies on the inhibition kinetics of plant protoporphyrinogen oxidase activities were done on a single biological system (etiolated maize seedlings). This enabled us to directly compare the effects of the inhibitors on the mitochondrial (mito.) and the etiochloroplast (etio.) enzymes. The maximum velocities of each enzyme ($V_{\max(\text{mito.})} = 5\text{--}6$ nmol of protoporphyrinogen oxidized/h per mg of protein and $V_{\max(\text{etio.})} = 6\text{--}7$ nmol of protoporphyrinogen oxidized/h per mg of protein) cannot be due to mutual contamination, since contamination was $< 10\%$, as indicated by the carotenoids and chlorophylls (markers of etiochloroplasts) or cytochrome *c* oxidase activity (marker of mitochondria) in each fraction. The maize mitochondrial and etiochloroplastic protoporphyrinogen oxidases are both competitively inhibited by DPEs, and the inhibition of the two enzymes is very similar. This is in good agreement with previous results (Jacobs & Jacobs, 1987), which reported that the protoporphyrinogen oxidases from barley etiochloroplasts and mitochondria had very similar molecular properties.

The difference in the reactivity of two plant protoporphyrinogen oxidases (from etiolated maize seedlings, mitochondria or potato tuber mitochondria) toward DPEs may not be surprising, since these plants are phylogenetically distant and their basic catalytic properties were different ($K_m = 0.42 \mu\text{M}$ in maize versus $0.09 \mu\text{M}$ in potato). This point was not further investigated.

The difference in reactivity of maize protoporphyrinogen oxidases *in vitro* toward AF and AFM is somewhat surprising, since AF is almost as efficient a herbicide as AFM *in vivo* (Becerril & Duke, 1989). This suggests that the active site of plant

protoporphyrinogen oxidase may be less accessible to water-soluble compounds or that other mechanisms, such as the charge present on AF or alterations in the stereochemistry of the molecules, could also affect binding to the active site. However, *in vivo*, physiological factors such as tissue permeability or metabolism (e.g. via the action of esterases converting AFM into AF) could control protoporphyrinogen oxidase inhibition.

The mouse liver protoporphyrinogen oxidase is also competitively inhibited by DPEs. The K_i values for the four DPEs tested are in the same range as those measured for the plant enzymes, except for RH, where the plant enzymes are more sensitive to inhibition than the mouse enzyme. Since all the inhibitions are competitive, the K_i determined in the present study allowed us to calculate theoretical IC_{50} values that correlated well with the experimental data. However, there were significant differences between the present IC_{50} values and our previously published data that prompted us to investigate this point further. The only obvious difference between the two sets of experiments was that two unrelated mouse strains were used for protoporphyrinogen oxidase measurements, i.e. Swiss mice in the present work and DBA/2 in our previous work; the mice were of the same age and were kept under the same diet, the livers were processed the same way and the enzyme assayed under standard conditions. In order to determine whether the increased sensitivity of DBA/2 to DPEs was due to modified catalytic properties of the enzyme in this strain, we measured its apparent Michaelis constant for protoporphyrinogen in the absence of inhibitor. The K_m was found to be identical with that measured with the Swiss-mouse liver enzyme ($0.24 \mu\text{M}$). However, the K_i for AFM (0.5 nM; results not shown) was consistent with the experimental IC_{50} . These results indicate that the two mouse strains have the same affinity for their substrate, but a different affinity toward the inhibitors. These differences between two strains of mice are puzzling, since in both cases the inhibition is strictly competitive, i.e. the substrate and the inhibitors share the same binding site. We may conclude that the binding sites of the substrate and the inhibitors are overlapping and that some biochemical or genetic factor (polymorphism) is involved in the binding of DPEs to mouse protoporphyrinogen oxidase.

The protoporphyrinogen oxidase from yeast mitochondrial membranes differs in two ways from the enzymes of other origins that we have examined. Its inhibition by DPEs is mixed competitive and not strictly competitive. This may be because the yeast *S. cerevisiae* is a facultative aerobic micro-organism which actually synthesizes some haem when grown under anaerobic conditions. This implies that protoporphyrinogen oxidase must be functioning in anaerobic cells. This activity is present either because the protoporphyrinogen oxidase has such a high affinity toward molecular oxygen that it functions with residual traces of oxygen present under anaerobiosis, or because protoporphyrinogen oxidase uses an electron acceptor other than oxygen for the anaerobic reaction. The mixed type of inhibition suggests that the DPEs interact with the enzyme in two ways or at two sites. This is more likely to occur with an enzyme able to catalyse a reaction in two different ways than with a single mechanism involving molecular oxygen as described for strictly aerobic organisms (plant or mouse). The second striking difference between yeast and plant or mouse enzymes lies in their reactivities toward AF. This water-soluble inhibitor is equally inhibitory as, or more inhibitory ($K_i = 7$ nM) than, the more lipid-soluble AFM ($K_i = 11$ nM), whereas AF is much less inhibitory than AFM (even less inhibitory than LS and RH for the plant enzymes) for all the other enzymes. This suggests that the active site of yeast protoporphyrinogen oxidase is more readily accessible to water-soluble chemicals than that of plant or mouse enzymes, indicating that there are important differences in

the topology of these enzymes. This possibility needs to be further investigated by using specific group reagents to probe the active sites of the enzymes and using radiolabelled DPEs to characterize the parameters of their binding to the various enzyme preparations and the mode of competition between the different inhibitors.

We thank Rhône-Poulenc Agrochimie (France) for supplying AFM and LS 820340, Rohm and Haas (Philadelphia, PA, U.S.A.) for RH 5348 and Dr. O. Parkes for his help in the preparation of the manuscript. This work was supported by grants from the Centre National de la Recherche Scientifique, Université Paris 7, and the Institut National de la Recherche Agronomique.

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Received 14 August 1990/19 November 1990; accepted 28 November 1990