Protoporphyrinogen IX-Oxidizing Activities Involved in the Mode of Action of Peroxidizing Herbicides†

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A plasma membrane (PM)-associated protoporphyrinogen oxidase (Protox)-like activity has recently been hypothesized to play a critical role in the oxidation of protoporphyrinogen IX exported by Protox-inhibited plastids to protoporphyrin IX in acifluorfen-methyl-treated plant tissues. Protox activities from etioplast and PM fractions from 7-day-old etiolated barley leaves were compared with regard to susceptibility to several Protox-inhibiting herbicides, effects of NADPH, quinones, and chelators, and other biochemical parameters. Etioplast Protox was much more susceptible to the herbicides than was PM Protox, whereas PM activity was much more inhibited by dithiothreitol (DTT). Cross-contamination could account for the relatively small effect of each of these inhibitors on that fraction on which they had little effect. NADPH was inhibitory to etioplast Protox activity; however, no inhibition was observed on PM Protox activity. Quinones such as duroquinone, juglone, or pyrroloquinoline quinone stimulated PM Protox activity, whereas lesser or no effects of these quinones were found in etioplasts. The K_m values for protoporphyrinogen IX of etioplast and PM Protox were 26 and 172 nM, respectively. DTT did not substantially change the K_m values in either preparation. Diethyldithiocarbamate, a copper chelator, strongly inhibited PM activity, while it had little or no effect on etioplast Protox. Hydrogen peroxide stimulated PM Protox activity, whereas cyanide ion and catalase inhibited it. There was much less effect of any of these compounds on etioplast Protox activity. These data further substantiate that PM Protox is different from etioplast Protox and that PM Protox is resistant to diphenyl ether herbicides. Moreover, they suggest that PM Protox has characteristics similar to those of a peroxidase.

Keywords: *Acifluorfen-methyl; peroxidase; peroxidizing herbicides; plasma membrane; protoporphyrinogen oxidase*

INTRODUCTION

Certain diphenyl ether and structurally related herbicides exert their herbicidal effect by causing rapid peroxidative photobleaching and desiccation of plant tissues (Kenyon et al., 1985; Kunert et al., 1987). They are known to be potent competitive inhibitors of protoporphyrinogen oxidase (Protox), the last common enzyme in the biosynthesis of both heme and chlorophylls (Matringe et al., 1989; Witkowski and Halling, 1989; Duke et al., 1990, 1991a,b; Scalla et al., 1990; Varsano et al., 1990; Camadro et al., 1991; Matringe et al., 1992b; Nandihalli et al., 1992a,b; Nandihalli and Duke, 1993). Paradoxically, inhibition of Protox causes massive accumulation of protoporphyrin IX (Proto IX), the product (rather than the substrate) of Protox (Matringe and Scalla, 1988; Becerril and Duke, 1989b; Matsumoto and Duke, 1990; Sherman et al., 1991a). The herbicidal effect correlates well with Proto IX accumulation (Matringe and Scalla, 1988; Becerril and Duke, 1989a,b; Matsumoto and Duke, 1990; Sherman et al., 1991b; Nandihalli et al., 1992c). This puzzling phenomenon of product accumulation has recently been explained by the existence of a herbicide-resistant Protox-like activity in the plasma membrane (PM) (Jacobs et al., 1991; Lee et al., 1993; Duke et al., 1994). When plastid Protox is inhibited by the diphenyl ether herbicide, protoporphyrinogen IX (Protogen IX), the substrate of Protox, is exported from the plastid (Jacobs and Jacobs, 1993) and is rapidly oxidized to Proto IX by PM-bound herbicide-resistant Protox-like activity (Jacobs et al., 1991; Lee et al., 1993). Proto IX, which is relatively lipophilic, accumulates in PM and/or other extraplastidic membranes (Duke et al., 1991b; Lee et al., 1993). In the presence of light and molecular oxygen, Proto IX generates singlet oxygen, subsequently causing rapid membrane lipid peroxidation and cellular death (Duke et al., 1990, 1991a; Scalla et al., 1990).

Little is known of the herbicide-resistant extraplastidic Protogen IX-oxidizing enzyme(s) of plants. Previous work has shown that such activity is found in PM-enriched barley root (Jacobs et al., 1991) and leaf (Lee et al., 1993) tissues. This activity is less substrate specific than plastid Protox, is more inhibited by reductants (except for NADPH) and diethyldithiocarbamate (DETC, a copper chelator), is more stimulated by certain quinones (juglone and duroquinone), and is relatively insensitive to plastid Protox inhibitors, such as diphenyl ether herbicides (Lee et al., 1993). Nothing else is known of the properties of this activity that appears to be required for the accumulation of Proto IX in plant cells treated with inhibitors of plastid Protox. In this paper we further characterize the PM-bound Protogen IX-oxidizing activity of barley leaves and contrast this activity with that of plastid Protox.

MATERIALS AND METHODS

Plant Material. Seeds of barley (*Hordeum vulgare* L. var. Post) were germinated in flats in a commercial greenhouse

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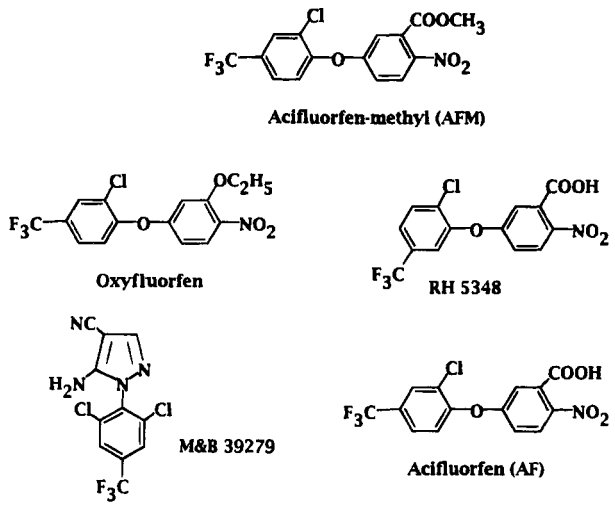

Figure 1. Structures of herbicides used in studies.

substrate (Jiffy-Mix; JPA, West Chicago, IL) and watered with distilled water. Plants were grown in darkness at 25 °C for 7 days.

Chemicals. Sources of chemicals were as follows: Dextran T 500 (Pharmacia LKB Biotechnology, Piscataway, NJ); salicylaldoxime (Eastman Kodak Co., Rochester, NY); juglone (Fluka Chemical Co., Ronkonkoma, NY); acifluorfen (AF) (ChemService, West Chester, PA); all other biochemicals except technical grade herbicides were from Sigma Chemical Co. (St. Louis, MO). Organic solvents were from Fisher Scientific (Norcross, GA) or Mallinckrodt Specialty Chemicals Co. (Chesterfield, MO). Technical grade herbicides acifluorfen-methyl (AFM), M&B 39279, oxyfluorfen, and RH 5348 (Figure 1) were provided by Rhône Poulenc AG Co., May & Baker Ltd., and Rohm and Haas Co., respectively.

Etioplast and PM Preparation. Etioplasts and PM fractions were obtained by slight modifications of the methods of Lee et al. (1993). These modifications resulted in higher yields of both fractions, and the specific Protogen IX-oxidizing activities of purified etioplast and PM fractions were increased 1.3- and 3-fold, respectively. All procedures were conducted under a dim, green light source. Leaves of dark-grown barley seedlings were cut into small pieces and homogenized with a Sorvall Omni-Mixer twice for 5 s at maximum speed using a fresh weight to volume ratio of 1:3. Homogenization buffer consisted of 50 mM HEPES (pH 7.8), 330 mM sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 2.5 mM dithiothreitol (DTT), and 0.1% BSA (fatty acid free). The homogenate was filtered through one layer of Miracloth (CalBiochem, La Jolla, CA) and was centrifuged at 200*g* for 5 min at 4 °C to remove crude cell debris. The resulting supernatant was centrifuged at 1500*g* for 20 min at 4 °C. The pelleted crude etioplasts were gently resuspended in suspension buffer using a small paintbrush. The suspension buffer was composed of 330 mM sucrose and 50 mM HEPES (pH 7.8). The resuspended crude etioplasts were further purified by layering over 35 mL of the suspension buffer containing 35% Percoll, in a 50 mL centrifuge tube and centrifuging at 8000*g* for 10 min in a Beckman JS-13 swinging bucket rotor at 4 °C. Intact etioplasts were recovered as a pellet, whereas broken etioplasts and other subcellular components formed a band at the top of the tube (Lee et al., 1991). The pelleted, purified etioplasts were resuspended in the same suspension buffer utilized in the resuspension of crude etioplasts. Etioplasts were stored at −80 °C until use.

A microsomal pellet was obtained by the differential centrifugation methods described by Kjellbom and Larsson (1984) and resuspended either in the suspension buffer or in a buffer consisting of 330 mM sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8) for the further purification of PM.

The isolation of PM by aqueous polymer two-phase partition was based on the methods of Larsson (1985) and Larsson et al. (1987). PM was purified by the partition of microsomal preparation in a dextran and poly(ethylene glycol) (PEG) two-phase system. The optimum phase composition was determined in preliminary experiments. Nine grams of the microsomal preparation (30−40 mg of protein) was added to 27 g of phase mixture to give a 36 g two-phase system with a final composition of 6.4% (w/w) dextran T 500, 6.4% (w/w) PEG 3350, 330 mM sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8). The phase system was thoroughly mixed, and phase settling was accelerated by centrifugation in a Beckman JS-13 swinging bucket rotor at 6000*g* for 5 min. The upper phase, where the PM had a high affinity, was repartitioned once against fresh lower phase to increase the purity of the PM. The final upper phase was diluted at least 3-fold using the suspension buffer and collected by centrifugation at 100000*g* for 45 min in a Beckman 60 Ti angle rotor. The resulting PM pellets were resuspended in the suspension buffer and stored at −80 °C until use.

Subcellular Marker Enzyme Assays. All enzymes were assayed at 25−26 °C. Activity was linear with respect to time and enzyme concentration. Spectrophotometric determinations were carried out on an SLM dual-wavelength spectrophotometer Model DW-2000 operated in the split beam mode. Activities of the marker enzymes were determined as before (Lee et al., 1993). The distribution of marker enzyme activities (Table 1) was almost the same as before (Lee et al., 1993). Latent IDPase and vanadate-inhibited K^+,Mg^{2+}-ATPase activities were much higher than previouly reported because of mistakes in calculations in the previous paper, but the relative distributions of the two enzymes were almost the same, except that the PM preparations in these studies were less enriched in vanadate-inhibited K^+,Mg^{2+}-ATPase.

Protox Assays. Before assay, the extracts of etioplasts and PM were thawed and sonicated twice for 5 s at 0 °C. Protein concentration was determined according to the method of Bradford (1976) with BSA as a standard, and the extracts were adjusted to 3 mg of protein/mL in the suspension buffer. When the test compounds (Figure 1) were utilized, they were added in a volume of 2 μL of acetone or DMSO to 200 μL of the extract. Acetone or DMSO was also added to control treatments. The extracts were allowed to incubate on ice for 15 min with or without the test compound.

Protogen IX was prepared according to the procedure of Jacobs and Jacobs (1982) with the following modifications. Proto IX stock solution (0.5 mM in 20% ethanol containing 10 mM KOH) was reduced to Protogen IX with approximately one-eighth volume of freshly ground sodium amalgam. The resulting colorless solution was adjusted to pH 8.0 by addition of an equal volume of (5× strength) assay buffer, consisting of 500 mM HEPES (pH 7.5) and 25 mM EDTA. Residual amalgam and porphyrin aggregates were removed by passing the solution through a 0.2−μm nylon syringe filter. DTT was added to the Protogen IX solution to a final concentration of 2 mM. The resulting preparation was stable in dim light at room temperature for at least 2 h.

Protox was assayed according to the procedure of Sherman et al. (1991a). The assay mixture consisted of 100 mM HEPES (pH 7.5), 5 mM EDTA, and approximately 2 μM Protogen IX. DTT was added to the assay at 2 mM concentration only when indicated. The reaction was initiated by addition of 0.1 mL of extract with or without the test compound to 0.9 mL of assay mixture and monitored for 2 min at 30 °C. Fluorescence was monitored directly from the assay mixture using a Shimadzu RF-5000U, temperature-controlled, recording spectrofluorometer with excitation and emission wavelengths set at 395 and 622 nm, respectively. The reaction rate was essentially constant over a 2 min period. Autoxidation of Protogen IX to Proto IX in the presence of heat-inactivated (80 °C for 15 min) extract was negligible. All treatments for Protox assays were duplicated or triplicated.

The exact concentration of Protogen IX for the K_m determinations was estimated by measuring Proto IX concentration after the Protogen IX was fully autoxidized to Proto IX in the air overnight. An appropriate amount of the autoxidized Protogen IX was diluted into 1 mL of 2.7 N HCl, and absorbance was measured at 554 nm. The concentration of Proto IX of the aliquot was quantified by using the extinction coefficient E_{mM} = 13.5 (Jacobs and Jacobs, 1982) and then assumed as a Protogen IX concentration.

Table 1. Specific Activities of Various Marker Enzymes in Crude and Purified Etioplasts, Microsomes, and PM from 7-Day-Old Etiolated Barley Leaves

		etioplast purification		PM purification	
marker	associated cell component	crude etioplasts	purified etioplasts	microsomes	PM
TPD[a]	etioplast	34.1 ± 1.5	132.2 ± 7.9	11.0 ± 2.3	8.3 ± 0.9
CCO[b]	mitochondria	70.8 ± 8.6	5.1 ± 1.8	36.0 ± 3.9	10.8 ± 1.6
HPR[c]	microbody	216.2 ± 31.4	31.1 ± 4.6	18.6 ± 2.1	9.6 ± 1.00
CCR[d]	ER	4.29 ± 0.21	0.24 ± 0.20	1.72 ± 0.34	0.70 ± 0.22
LDH[e]	cytosol	16.5 ± 1.5	1.42 ± 0.41	19.3 ± 0.24	2.75 ± 2.01
IDPase[f]	Golgi	325.9 ± 11.0	71.3 ± 9.5	490.8 ± 9.4	159.0 ± 22.8
ATPase[g]	PM	196.7 ± 14.8	9.9 ± 1.2	249.5 ± 26.6	585.2 ± 28.9

[a] NADP:triose phosphate dehydrogenase [μmol min^{-1} (mg of protein)$^{-1}$]. [b] Cyt c oxidase [μmol min^{-1} (mg of protein)$^{-1}$]. [c] Hydroxypyruvate reductase [μmol min^{-1} (mg of protein)$^{-1}$]. [d] Antimycin A-insensitive Cyt c reductase [μmol min^{-1} (mg of protein)$^{-1}$]. [e] Lactate dehydrogenase [μmol min^{-1} (mg of protein)$^{-1}$]. [f] Latent IDPase [nmol min^{-1} (mg of protein)$^{-1}$]. [g] Vanadate-inhibited K^+,Mg^{2+}-ATPase [nmol min^{-1} (mg of protein)$^{-1}$].

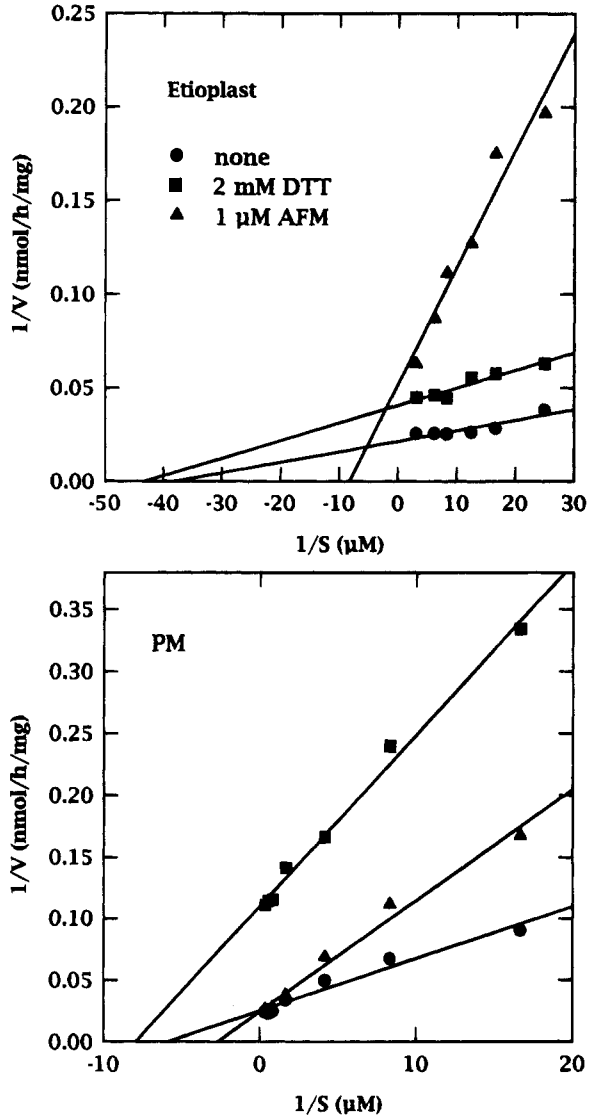

Figure 2. Lineweaver–Burk plots of etioplast and PM-bound Protogen IX-oxidizing activities with or without 2 mM DTT or 1 μM AFM.

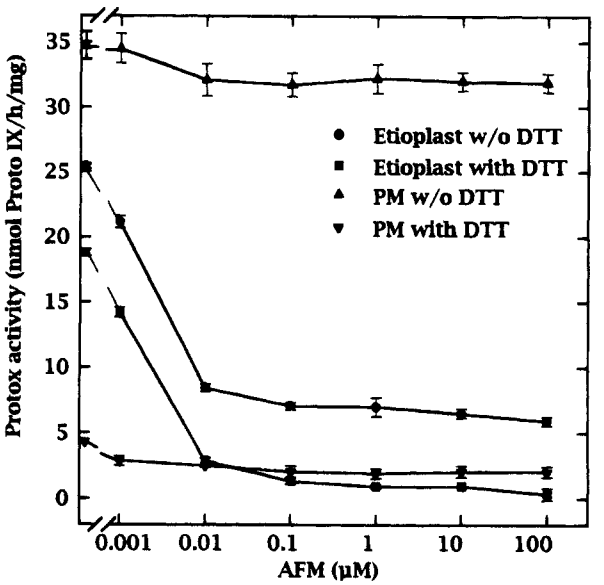

Figure 3. Dose–response plots for AFM inhibition of etioplast and PM-bound Protogen IX-oxidizing activities with or without 2 mM DTT. The data points nearest the left Y axis are control (no AFM) values. Error bars are ±1 SE of the mean.

RESULTS

K_m Values. Lineweaver–Burk plots for etioplast and PM-bound Protogen IX-oxidizing activities indicated K_m values of 26 and 172 nM, respectively, in the absence of reductant (Figure 2). Inclusion of 2 mM DTT had little effect on the K_m values. The Lineweaver–Burk plots with and without 1 μM AFM indicated that AFM was a competitive inhibitor of both etioplast Protox and PM-bound Protox-like activity. However, the inhibitory effect of AFM on PM activity was observed only at a very low Protogen IX concentration range (0.06–0.24 μM). With higher concentrations of Protogen IX, the AFM inhibition of PM activity was not significant. This inhibition might be the result of contamination of the PM fraction with etioplast or etioplast Protox (see Discussion).

Herbicide Effects. Complete dose–response profiles for AFM on purified etioplast and PM preparations with or without 2 mM DTT revealed that virtually all etioplast Protox activity was eliminated by 10 μM AFM under reducing conditions, whereas there was very little (*ca.* 8%) inhibition of PM Protogen IX-oxidizing activity under nonreducing conditions (Figure 3). Similar results were obtained with other Protox inhibitors (Figure 4). The intracellular reducing environments of the PM and plastid are low and high, respectively [see Lee et al. (1993) for discussion of this]. In previous work with crude plastids (6000g pellet) we found AF and RH 5348 to be much less active than AFM, with Protox I_{50} values of 4.0, 3.9, and 0.04 μM, respectively (Nandihalli et al., 1992a). The relatively high (*ca.* 80%) inhibition of purified etioplast Protox activity by 1 μM AF and RH 5348 in the present studies could be due to lack of contamination of purified etioplast by PM and/or the different barley variety that we used in this study.

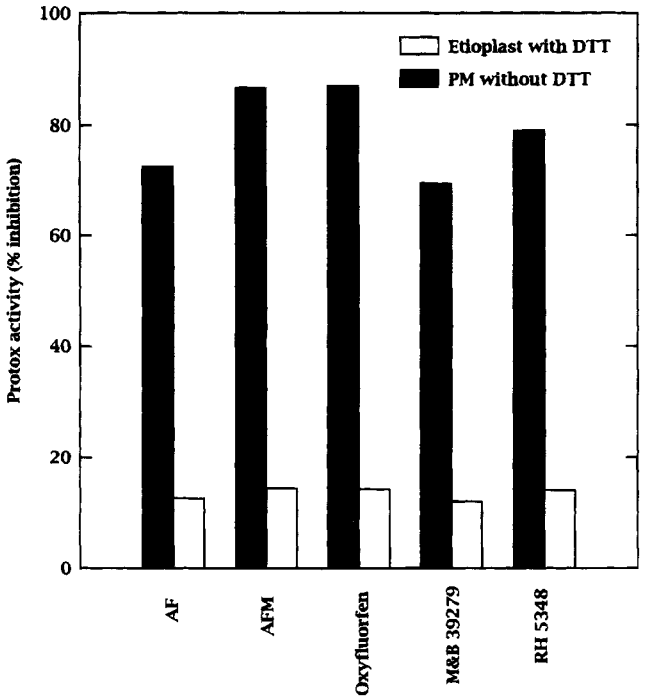

Figure 4. Inhibitory effects of six Protox inhibitors on etioplast and PM Protogen IX-oxidizing activities. Protox inhibitors were 1 μM in the assay mixture. Etioplast assays were run in the presence of 2 mM DTT, whereas PM assays were not. SEs of the mean averages are less than 12% of the mean.

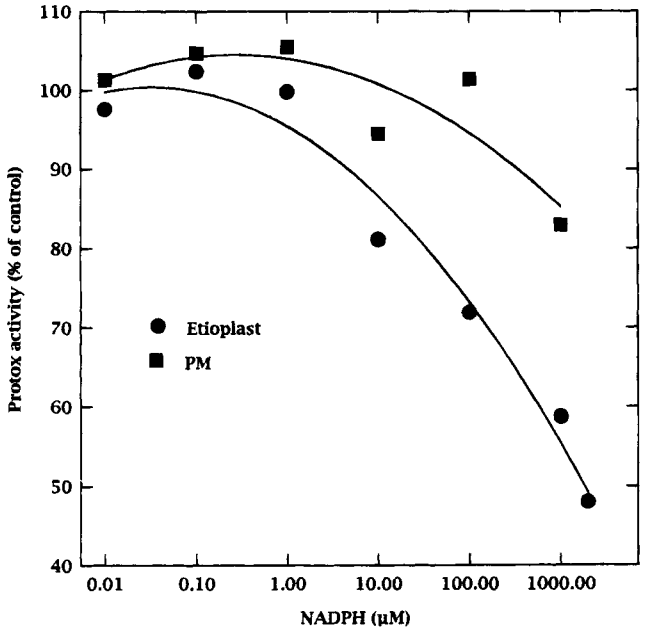

Figure 5. Effects of NADPH on Protogen IX-oxidizing activities of etioplast and PM. SEs of the mean averages are less than 4% of the mean.

NADPH Effects. A complete dose–response of NADPH effects on PM and etioplast Protogen IX-oxidizing activities confirmed that NADPH inhibited etioplast activity while having no effect on PM activity except at 1 mM (Figure 5).

Quinone Effects. We reported previously that the quinones duroquinone and juglone stimulate the activity of PM-mediated Protogen IX oxidation more than they do etioplast Protox activity (Lee et al., 1993). Pyrroloquinoline quinone (PQQ), a naturally occurring component of some quinoprotein enzymes, stimulated only the

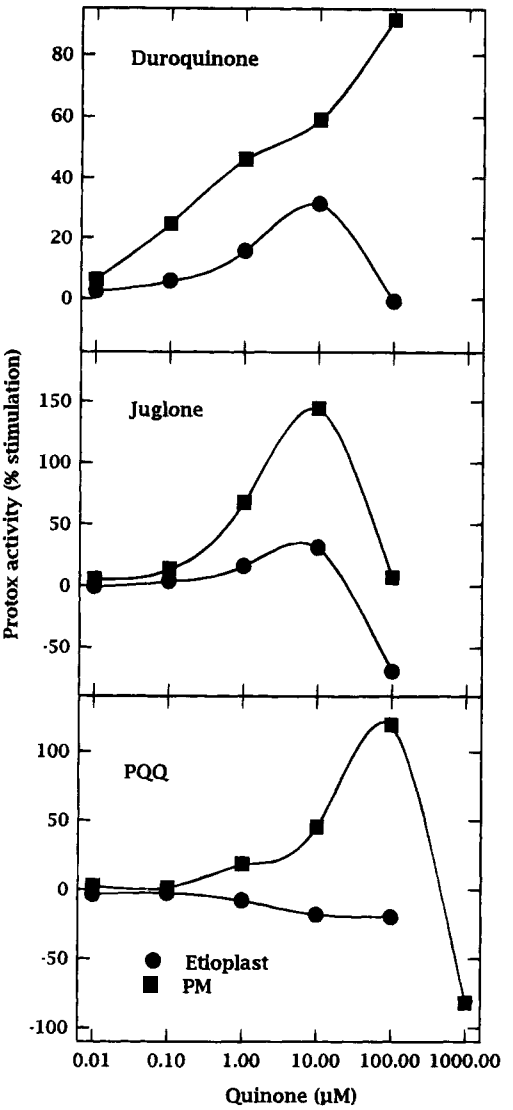

Figure 6. Effects of three different quinones on Protogen IX-oxidizing activities of etioplast and PM. Nonenzymatic effects of the quinones (activity in a heated preparartion) were subtracted from the enzymatic activities before calculation. SEs of the mean averages are less than 10% of the mean.

PM activity (Figure 6). Other quinones tested at 100 μM, decylplastoquinone, coenzyme Q_0, and vitamin K_1, had no significant stimulatory effect on Protogen IX-oxidizing activity of either preparation (data not shown). NADPH did not significantly influence the effect of juglone on PM-bound activity (data not shown).

Chelators and Cu^{2+} Effects. Complete dose–response curves for DETC, a copper chelator, effects on PM and etioplast Protogen IX-oxidizing activities confirmed that PM-bound Protox-like activity was more sensitive to the inhibitory effects of this compound than was etioplast Protox (Figure 7A). Salicylaldoxime, another copper chelator (Golbeck, 1980), had a stimulatory effect on PM activity at higher concentrations (Figure 7B). Much lower stimulation of PM activity was obtained by salicylic acid than by salicylaldoxime (Figure 7B,D). However, salicylic acid at concentrations up to 1 mM had no effect on etioplast activity (Figure 7D). The calcium chelator ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid and the iron chelator thenoyltrifluoroacetone at concentrations up to 1 mM had no effect on either activity (data not shown). Another iron chelator, *o*-phenanthroline, at 0.1 mM slightly stimulated activities of both preparations (data

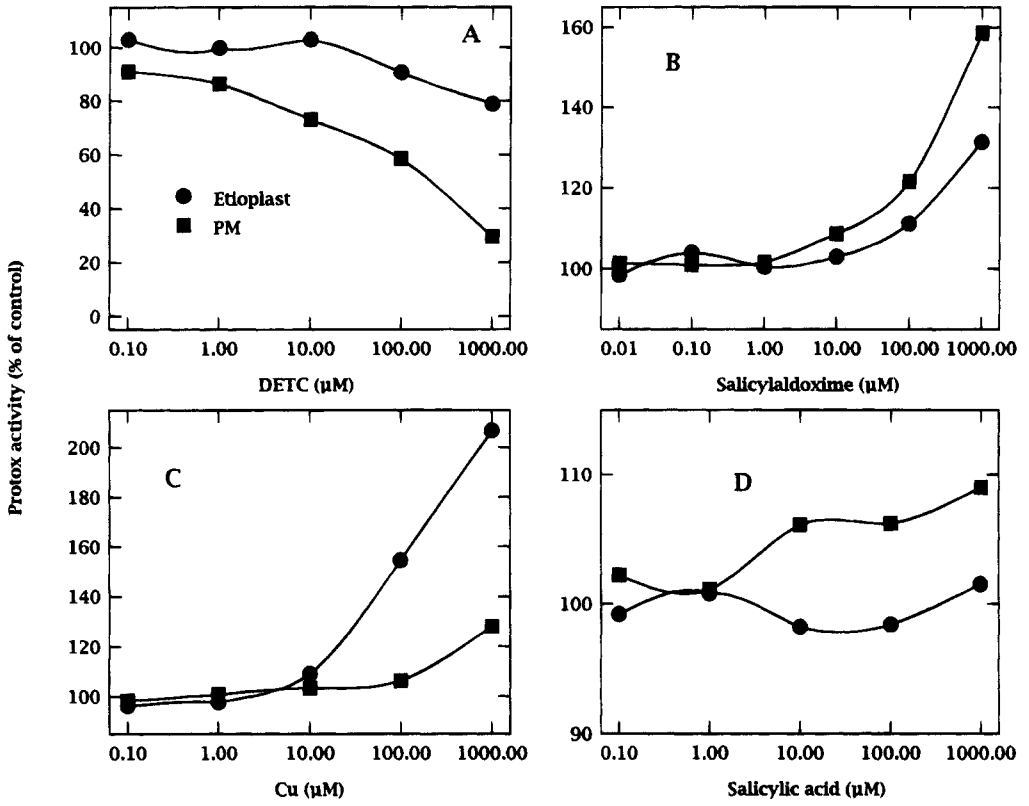

Figure 7. Effects of DETC (A), salicylaldoxime (B), Cu^{2+} (C), and salicylic acid (D) on Protogen IX-oxidizing activities of etioplast and PM. SEs of the mean averages are less than 16% of the mean.

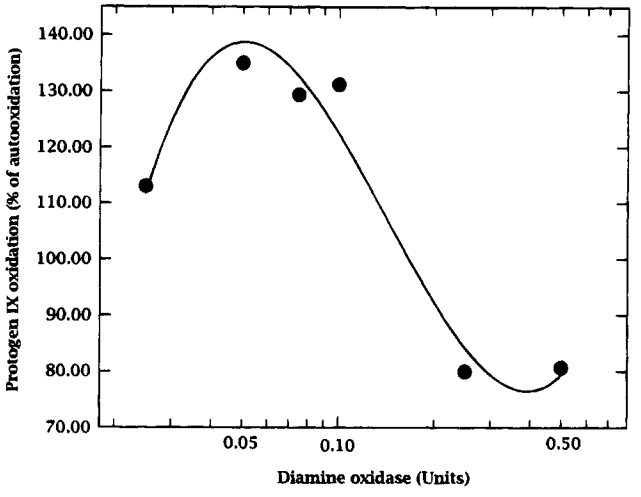

Figure 8. Protogen IX-oxidizing activity of diamine oxidase. One unit of diamine oxidase oxidizes 1 μmol of putrescine/h at pH 7.2 at 37 °C.

not shown). Cu^{2+} alone, within a narrow concentration range, stimulated activity of both preparations; however, the effect was more pronounced in the etioplast preparation (Figure 7C).

Diamine Oxidase and Diamines. Protox could be considered a diamine oxidase because it oxidizes two amines of the porphyrin macrocycle. Porcine diamine oxidase was found to have only a small amount of Protox-like activity within a narrow concentration range (Figure 8), but it was very different from the range of autoxidation of Protogen IX to Proto IX. The diamines putrescine and cadaverine had no effect on either PM-bound or etioplast Protogen IX-oxidizing activity (data not shown).

Carbonyl Reagent Effects. A complete dose–response of the effects of phenylhydrazine on Protogen

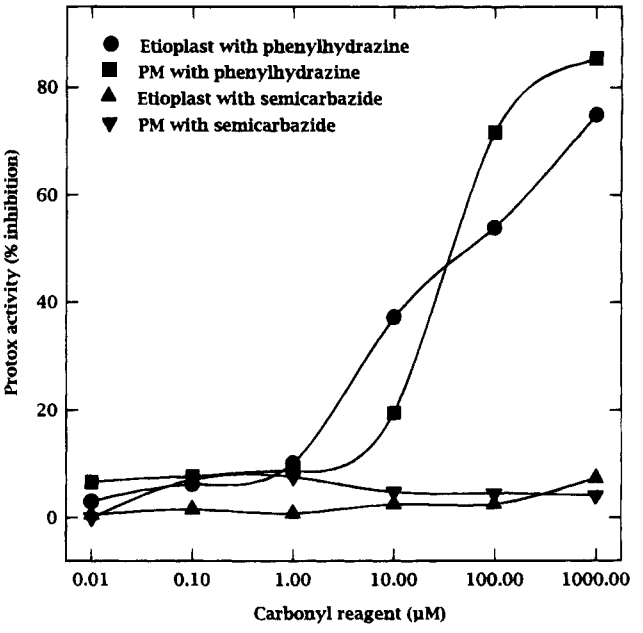

Figure 9. Effects of two carbonyl reagents on Protogen IX-oxidizing activities of etioplast and PM. SEs of the mean averages are less than 6% of the mean.

IX-oxidizing activity of each of the preparations indicated that either activity was equally inhibited by this compound, whereas semicarbazide had no effect on either activity (Figure 9).

Electron Acceptors and Electron Transport Inhibitors. Quinacrine, a compound known to interrupt transmembrane redox activities (Brightman et al., 1988), at 10 μM inhibited PM-bound Protox-like activity by almost 20% (data not shown). At the same concentration, ferricyanide had no effect, and 2,6-dichlorophenolindophenol reduced activity by 35% (data not shown).

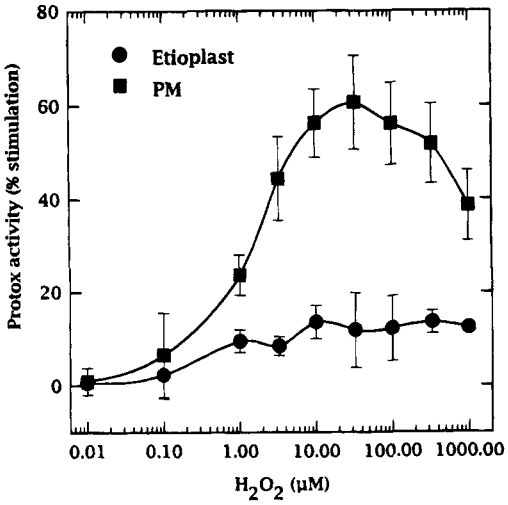

Figure 10. Effects of H_2O_2 on Protogen IX-oxidizing activities of etioplast and PM. Error bars are ±1 SE of the mean.

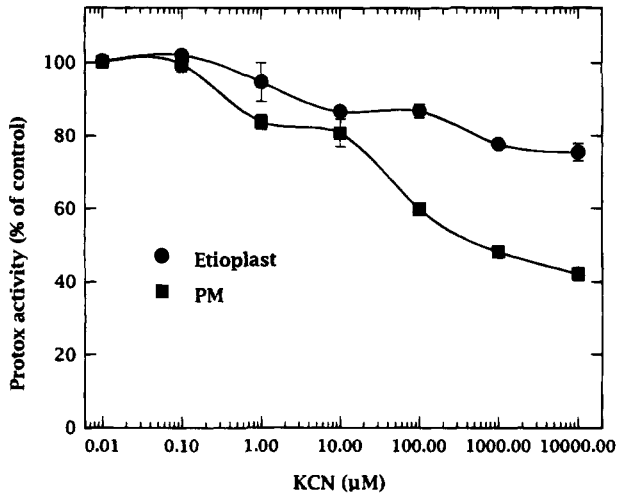

Figure 11. Effects of KCN on Protogen IX-oxidizing activities of etioplast and PM. Error bars are ±1 SE of the mean.

H_2O_2, CN^-, and Catalase. Compounds known to affect peroxidase activity had more effect on PM Protogen IX-oxidizing activity than on etioplast activity (Figures 10–12). H_2O_2 stimulated the activity of PM Protogen IX-oxidizing activity by 60%, but had little effect on etioplast Protox at any concentration (Figure 10). CN^- reduced PM Protogen IX-oxidizing activity by almost 60% at 10 mM, but etioplast activity was reduced by only about 20% at this concentration (Figure 11). Catalase had no effect on etioplast Protox, while it reduced PM Protogen IX-oxidizing activity by more than 50% at 3000 units/mL (Figure 12).

DISCUSSION

The etioplast and PM Protox K_m values for Protogen IX (26 and 172 nM, respectively) indicate that the etioplast enzymes bind Protogen IX more efficiently than the PM enzymes. This is not suprising, as it is unlikely that the PM activity has a true Protox function *in vivo*. This also indicates that the PM-bound Protox-like enzyme(s) could be functional as a Protox only after sufficient Protogen IX accumulates in its vicinity. Etioplast Protox is apparently more tightly regulated. There are few published K_m values for Protox. Camadro et al. (1994) have reported K_m values of 300 and 70 nM for highly purified lettuce etioplast and yeast Protox,

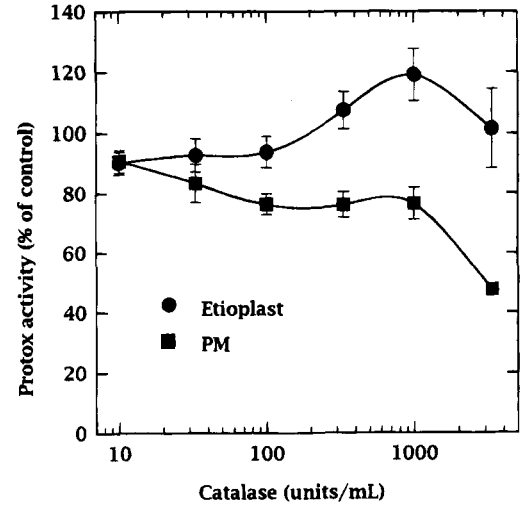

Figure 12. Effects of catalase on Protogen IX-oxidizing activities of etioplast and PM. Error bars are ±1 SE of the mean. One unit of catalase decomposes 1 μmol of H_2O_2/min at pH 7 at 25 °C.

respectively. Removal of Protox from its membrane site might be expected to increase the K_m value.

DTT had little effect on K_m values in either preparation (Figure 2), even though it reduced the activity of the PM substantially. This suggests that the effect of DTT is not a direct effect on enzyme efficiency but is due to another effect, such as availability of an electron acceptor. The Lineweaver–Burk plots with AFM, when compared to those without AFM, indicate competitive inhibition of AFM with Protogen IX. This is consistent with previous binding studies (Varsano et al., 1990; Camadro et al., 1991) and what is known of the structural similarities between Protogen IX and Protox inhibitors (Nandihalli et al., 1992a,b; Nandihalli and Duke, 1993, 1994).

Approximately the same amount of Protogen IX-oxidizing activity is inhibited by DTT, regardless of the AFM concentration in either PM or etioplast preparation (Figure 3). Other reductants, except for NADPH, have previously been shown to behave like DTT with respect to PM-bound and etioplast Protogen IX-oxidizing activities (Lee et al., 1993). There appears to be no interaction between AFM and DTT, supporting the view that DTT is not affecting the active site of Protox. The much larger effect of DTT on the activity of the PM than on etioplast Protox activity suggests that PM activity may be much more dependent on the availability of an electron acceptor that is affected by DTT. It is clear from Figure 3 that most of the Protox-like activity of the PM that is inhibited by DTT is insensitive to AFM. The same appears to be true for the etioplast. The relatively small amount of DTT-inhibited Protox activity of the etioplast preparation could thus be due to contamination with PM or PM Protox-like activity. Conversely, the relatively small amount of PM activity inhibited by AFM could be due to etioplast or etioplast Protox contamination. The marker enzyme activity data (Table 1) indicate that the preparations are purer than this, but there is no guarantee that the etioplast Protox and the Protox-like activity of the PM cosegregate precisely with the respective marker enzymes. For example, plastid Protox is predominantly associated with the plastid envelope (Matringe et al., 1992a), but the etioplast marker NADP:triose phosphate dehydrogenase is not exclusively envelope associated (Quail, 1979).

We have previously speculated that the strong reducing environment of the plastid relative to the PM results in maximal inhibition of etioplast Protox and minimal inhibition of PM Protox-like activities (Lee et al., 1993). If the effects of DTT on etioplast preparations and the effects of AFM on PM preparations are due to cross-contamination, this argument may be irrelevant.

We (Lee et al., 1993) and Tietjen (1991) showed previously that quinones stimulate Protox activity. Tietjen indicated that this was an effect on etioplast Protox; however, his assay was with a crude etioplast preparation. Our data in this and the previous paper indicate that the effect is primarily on the PM-bound activity. Again, this suggests that this activity is more dependent on external oxidizing compounds than etioplast Protox. PQQ was an excellent stimulator of PM activity (Figure 6). PQQ is a ubiquitous natural compound that is a cofactor of many redox enzymes called quinoproteins (Davidson, 1993). Many quinoproteins are copper-containing enzymes.

The copper chelator DETC had a strong effect on the PM-bound activity, whereas there was relatively little effect on etioplast Protox activity (Figure 7A). The effect of DETC was near maximal at 1 mM in both preparations, with about 20% inhibition of the etioplast Protox and 80% inhibition of the PM-bound activity. This is comparable to the effects of DTT on the two preparations (Figure 3), suggesting as before that there could be 20% cross-contamination of the two preparations. The effect of DETC might not be related to copper chelation, since another reported copper chelator, salicylaldoxime (Golbeck, 1980), had a stimulatory effect (Figure 7B). However, DETC has the ability to actually remove copper from some enzymes (Calabrese et al., 1976; Skotland and Ljones, 1979), and we are unaware of any such claims for salicylaldoxime. Alternatively, salicylaldoxime may have another function in the cell in addition to copper chelation. Salicylic acid, a natural signal molecule for the induction of disease resistance and thermogenesis (Raskin, 1992), has recently been reported to enhance the generation of H_2O_2 in tobacco leaf tissues (Chen et al., 1993). Whether salicylaldoxime also causes *in vivo* H_2O_2 generation is unknown. In this study, however, salicylaldoxime stimulated PM Protogen IX-oxidizing activity more than salicylic acid (Figure 7B,D). The questions of whether stimulation of PM Protox-like activity by salicylaldoxime is mediated by elevated H_2O_2 concentration and, if so, how H_2O_2 stimulates PM activity, need to be answered.

The 100% stimulatory effect of 1 mM Cu^{2+} on Protox activity of the etioplast (Figure 7C) was unexpected. Some copper-containing enzymes can be activated by adding copper (Skotland and Ljones, 1979). However, addition of copper to copper-deficient plants does not always restore the enzymes (Delhaize et al., 1985). There was a 30% stimulation of PM activity at the same optimal concentration. Again, this suggests 20% contamination with etioplast or etioplast Protox. These results seem to be inconsistent with the DETC results. A possible reconciliation of the data is that the copper associated with etioplast Protox cannot be removed by DETC but that associated with the PM-bound Protox-like activity can be.

If one assumes that the PM-bound enzyme is a copper protein, a hint regarding the identity of the enzyme is given, provided the activity that we are measuring is associated with a known enzyme. There are several known copper-containing redox enzymes in plant cells: polyphenol oxidase, superoxide dismutase, and polyamine oxidases. Polyphenol oxidase is known to occur only in the plastid in healthy plant cells (Vaughn et al., 1988), and copper-containing superoxide dismutase is also predominantly found in the plastid (Salin, 1988). Polyamine oxidases have been reported to be associated with cell walls (Federico et al., 1992), and they are quinoproteins (Davidson, 1993). The Protogen IX molecule has two amino groups to be oxidized to form Proto IX. Theoretically, Protox could be considered a diamine oxidase, a copper-containing quinoprotein with a topaquinone cofactor (Duine, 1991). However, Protogen IX contains secondary amino groups. Since diamine oxidases preferentially oxidize primary amino groups of aliphatic amines and polyamines (*e.g.*, spermidine and spermine) to secondary amino groups (Cogoni et al., 1990), the lack of significant Protox activity of a commercially available diamine oxidase (Figure 8) and lack of effect of two diamines on PM Protox-like activity were not unexpected. Furthermore, the possibility that the PM Protogen IX-oxidizing activity is associated with a diamine oxidase could be ruled out since the carbonyl reagents phenylhydrazine and semicarbazide did not differentially inhibit Protogen IX-oxidizing activities from etioplast and PM (Figure 9). Carbonyl reagents have routinely been used to identify the organic prosthetic group of amine oxidase as a quinoid cofactor (Duine, 1991; Davidson, 1993). No protein-associated quinoid cofactor could be detected in active, purified murine Protox (Proulx and Dailey, 1992). Therefore, the stimulatory effect of quinones on PM Protogen IX-oxidizing activity (Figure 6) might be explained by the fact that quinones act as an alternative or additional electron acceptor to molecular oxygen (Tietjen, 1991; Lee et al., 1993) rather than as a cofactor of the enzyme during the oxidation of Protogen IX to Proto IX , a six-electron transfer reaction. Bacterial Protox uses electron acceptors other than molecular oxygen (Jacobs and Jacobs, 1979, 1981) and is resistant to diphenyl ether herbicides (Jacobs et al., 1990). However, no such stimulatory effect of quinones was found in etioplast Protox activity (Figure 6). Thus, like animal Protox (Ferreira and Dailey, 1988), etioplast Protox apparently used only molecular oxygen as an electron acceptor. The stimulatory effect of H_2O_2 on PM activity, but not on etioplast activity (Figure 10), could be explained similarly. Molecular oxygen is apparently not limiting in our *in vitro* Protox assay conditions. The reason reductants, except NADPH, have high inhibitory effects on PM activity (Lee et al., 1993; Figure 3) could be explained by PM activity requiring electron acceptors other than molecular oxygen that can also accept electrons from reductants.

The results with H_2O_2, CN^-, and catalase (Figures 10–12) suggest that the PM Protox-like activity could be associated with a peroxidase and are inconsistent with the view that the activity is associated with a diamine oxidase. We intend to further explore the similarity of this enzyme with peroxidase in future research. The results reported here clearly demonstrate, however, that the Protox-like enzymatic activity associated with the PM of plant cells is unlike the herbicide-sensitive etioplast Protox and is responsible for the massive accumulation of Proto IX in plant tissues treated with diphenyl ether herbicides.

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