HPLC and *in vivo* Spectrophotometric Detection of Porphyrins in Plant Tissues Treated with Porphyrinogenic Herbicides

Stephen O. Duke, Mary V. Duke, and Hee Jae Lee

Southern Weed Science Laboratory, U.S. Department of Agriculture, Agricultural Research Service, P.O. Box 350, Stoneville, Mississippi 38776, U.S.A.

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Protoporphyrinogen oxidase (Protox) inhibitors and other compounds which block or stimulate the porphyrin pathway can cause sufficient levels of porphyrins to accumulate in plant tissues for severe photodynamic damage to occur. The gross symptomology for all of these porphyrinogenic herbicides is similar. Porphyrin accumulation induced by three porphyrinogenic herbicides acifluorfen (AF), δ-aminolevulinic acid (ALA), and 2,2′-dipyridyl (DY) was determined by *in vivo* spectrophotometry and HPLC methods. The averaged *in vivo* difference spectra between untreated and AF-treated (30 μM for 20 h in darkness) yellow cucumber cotyledon discs approximated the absorption spectra of protoporphyrin IX (Proto IX). There was also an enhanced peak near 503 nm. Treatment of cotyledon discs with ALA alone generated a difference spectrum of protochlorophyllide (PChlide) in combination with Mg-Proto IX or Mg-Proto IX monomethyl ester (Mg-Proto IX ME). With ALA and AF in combination, the PChlide and Mg-Proto IX portions of the difference spectrum were reduced and the Proto IX peak and peak near 503 nm were increased. DY treatment yielded a difference spectrum with peaks approximating those of Proto IX and Mg-Proto IX ME, along with a peak near 503 nm. The presence of all porphyrins detected by *in vivo* spectrophotometry except for the 503 nm peak was confirmed with HPLC. Proto IX monomethyl ester was found by HPLC to be especially elevated in treatments with AF. The *in vivo* 503 nm peak and *in vitro* studies with Protox-containing barley etioplast preparations suggest that prototetrahydroporphyrin IX (an oxidation state intermediate between protoporphyrinogen IX and Proto IX) may accumulate under some conditions. These data demonstrate that rapid *in vivo* spectrophotometric studies can provide much of the qualitative results of HPLC studies and can confirm that *in vitro* results correspond with the *in vivo* situation.

Introduction

Certain substituted diphenyl ether herbicides such as acifluorfen (AF) are strong inhibitors of protoporphyrinogen oxidase (Protox), the last enzyme common to the synthesis of both chlorophyll and heme [1–5]. These compounds are competitive inhibitors and are analogs of one half of the Protox substrate, protoporphyrinogen IX (Protogen IX), molecule [1, 6–8]. Inhibition of Protox by these herbicides apparently causes the enzyme product, protoporphyrin IX (Proto IX) to accumulate [9–11], as it does in yeast or humans with dysfunctional Protox [12, 13]. Protogen IX is relatively labile and can autooxidize to Proto IX. Therefore, it has been assumed that the Proto IX formed in tissues in which Protox activity is absent, either through herbicidal inhibition or genetic defect, is due to autooxidation of the substrate outside the normal porphyrin pathway [4, 14–17].

In plants, the most active site of porphyrin synthesis in leaf cells is the plastid, and the plastid envelope is enriched in Protox [18]. In plant cells treated with Protox-inhibiting herbicides, Proto IX appears to accumulate largely outside the plastid, either in the plasma membrane or cell wall [16, 19]. Furthermore, a Protox-like activity appears to be associated with the plasma membrane [20]. This activity is not inhibited significantly by Protox-inhibiting herbicides. Whether this extraorganellar Protogen-oxidizing activity is enzymatic is still not clear. These previous data indicate that inhibition of plastidic Protox leads to export of Protogen IX by the plastid, with subsequent oxidation to Proto IX at the plasma membrane.

Abbreviations: AF, acifluorfen; AFM, acifluorfen-methyl; ALA, δ-aminolevulinic acid; DY, 2,2′-dipyridyl; Mg-Proto IX ME, Mg-protoporphyrin IX monomethyl ester; PChlide, protochlorophyllide; Proto IX, protoporphyrin IX; Protogen IX, protoporphyrinogen IX; Proto IX ME, protoporphyrin IX monomethyl ester; Protox, protoporphyrinogen oxidase; PTP IX, prototetrahydroporphyrin IX.

Reprint requests to Stephen O. Duke.

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We have demonstrated dramatic increases in extractable Proto IX from AF or AFM-treated plant tissues within a half hour or less after exposure to the herbicide in the light [21, 22]. However, in both of these studies, herbicidal effects lagged hours behind Proto IX accumulation. The present study suggests that some of the lag could be due to rapid *in vivo* accumulation of Protogen IX or oxidative intermediates instead of Proto IX. These compounds might be converted to Proto IX rapidly during extraction and sample preparation or more slowly *in vivo*.

In a recent paper it was demonstrated that *in vivo* spectrophotometric methods could be used to detect accumulation of Proto IX in AF-treated plant tissues [23]. This can be done in tissues with very low chlorophyll levels, such as those treated with tentoxin, a compound which arrests chloroplast development [24]. These tissues retain the capacity for high levels of plastid porphyrin synthesis in darkness [19, 24]. Lack of high chlorophyll levels allows the detection of porphyrin pathway intermediates by *in vivo* spectrophotometry.

Porphyrinogenic herbicides other than Protox inhibitors, such as δ-aminolevulinic acid (ALA) and 2,2′-dipyridyl (DY), have been the focus of studies [25, 26]. *In vivo* spectrophotometric methods could be used to probe their action also.

In the present paper we provide further evidence that Proto IX, protoporphyrin IX monomethyl ester (Proto IX ME), and, possibly, the oxidation intermediate of Protogen IX to Proto IX, prototetrahydroporphyrin IX (PTP IX), accumulate in plant tissues exposed to the Protox inhibitor AF. PTP IX has been reported to have a 503 nm absorption maxima that is not shared by other porphyrin intermediates, and its accumulation has been considered to be the result of non-enzymatic oxidation of Protogen IX [27–29]. Furthermore, findings in this paper confirm that much of the Proto IX extracted from AF-treated tissues exists as Proto IX *in vivo* and is not due to chemical oxidation of accumulated Protogen IX during extraction. Finally, the *in vivo* spectrophotometric and HPLC results agreed in all treatments with porphyrinogenic herbicides, indicating that *in vivo* spectrophotometry is a reliable, rapid method for predicting whether a herbicide acts by porphyrinogenesis and, if so, predicting its site in the porphyrin pathway.

Materials and Methods

Plant material

Seeds of cucumber (*Cucumis sativus* L., *cv.* Straight Eight) were imbibed in 80 μM tentoxin (Sigma Chemicals, St. Louis, MO) for 24 h before planting as previously described [19, 30]. The seeds were then planted in flats in a commercial greenhouse substrate (Jiffy-Mix; JPA, West Chicago, IL) and watered with distilled water.

Plants were grown at 25 °C for 7 to 8 days under 150 μmol m^{-2} s^{-1} photosynthetically active white light and >90% relative humidity before cotyledons were harvested for use. Treating with tentoxin produces yellow cotyledons that are morphologically normal, but contain little or no chlorophyll [19, 24, 30].

Tissues were treated with AF as before [31] by cutting 50, 4 mm diameter cotyledon or leaf discs with a cork borer and then placing them in a 6 cm diameter polystyrene petri dish in 5 ml of 1% sucrose, 1 mM MES (pH 6.5) medium with or without test compounds. Technical-grade treatment compounds were used: AF {5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid}, a gift of Rohm and Haas Co.; DY and ALA were purchased from Sigma Chemicals. The discs were then incubated at 25 °C in darkness for 20 h.

Barley seed (*Hordeum vulgare* L., *cv.* Morex) for membrane and Protox preparations were germinated and grown in 1.2 L pots in darkness at 25 °C for 7 days as before [6].

In vivo spectrophotometry

All procedures were carried out under dim, green light. *In vivo* spectral scans were performed with a dual wavelength/dual beam spectrophotometer (Model DW-2000, SLM Instruments, Urbana, IL) in the dual beam mode. Cotyledon discs of cucumber (described above) were taped with translucent, neutral-density tape to flat-black, anodized aluminum plates to completely cover an orifice which registered with the measuring beam of the spectrophotometer, which was focused on the orifice with a microbeam attachment. The reference beam was focused on a reference orifice which was covered with tape only. The sample-holding plates were placed within 1 cm of the photomultiplier tube. Only one disc was measured at a time with the method. Several scans were made of

each treatment and then an average of the computer-stored scans was generated. Average values of untreated discs were subtracted from average values of treatments to obtain difference spectra to evaluate the treatment effects.

To better determine the underlying absorption maxima of the various compounds contributing to the *in vivo* spectra, the spectra were analyzed with a computer program (PeakFit, Jandel Scientific, San Rafael, CA) designed to deconvolute complex absorption spectra into their Gaussian components without user input to suggest particular peaks. The computer-generated peaks did not absolutely correspond with the spectral peaks of standards of chlorophyll intermediates in solution. However, absorption spectra of these somewhat lipophilic compounds in crude membrane preparations (see below) were obtained to more closely simulate their *in vivo* microenvironment (Table I).

All procedures for preparation of crude membrane extracts were carried out under dim, green light, and the preparation was kept at 0–4 °C, except where noted. Six-day-old, tentoxin-treated cucumber cotyledons were homogenized by mortar and pestle in a buffer containing 0.25 M sucrose, 25 mM Tris-MES, and 3 mM EDTA, pH 7.2 [32], at a ratio of 0.25 g of tissue per milliliter of buffer. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 150 × *g* for 1 min to remove cell debris that might settle to the bottom of the cuvette during spectra scanning. The supernatant was kept on ice until use. Porphyrin standards were prepared in 100% HPLC-grade methanol at 25 μM and added to the membrane preparation to produce a final concentration of 2.5 μM. A blank containing 10% methanol in cucumber extract was also prepared. All preparations were allowed to equilibrate to room temperature before measurement.

HPLC of porphyrins

Porphyrins were extracted and identified by HPLC as before [33]. All extractions for HPLC were made under a dim, green light source. Samples (50 cotyledon discs) were homogenized in 3 ml of HPLC-grade methanol: 0.1 N NH_4OH (9:1, v/v) with a Brinkmann Polytron at 60% full power for 15 s. The homogenate was centrifuged at 30,000 × *g* for 10 min at 0 °C and the supernatant was saved. The pellet was resuspended in 3 ml of methanol, sonicated for 5 min, and centrifuged at 30,000 × *g* for 10 min at 0 °C. Supernatants were combined and evaporated to dryness at 40 °C with a rotary evaporator. The residue was dissolved in 2 ml of HPLC-grade basic methanol, sonicated for 5 min, and filtered through a 0.2 μm syringe filter. Samples were stored in light-tight glass (wrapped in aluminum foil) vials at −20 °C until analysis by HPLC.

HPLC determinations were made with a system composed of Waters Associates components which included: two Model 510 pumps; a Model 712 autosampler; a Maxima 820 controller a Model 990 photodiode spectrophotometric detector; and a Model 470 fluorescence detector. The column was a 250 × 4.6 mm (i.d.) Spherisorb 5 μm ODS-1 reversed phase column preceded by a Bio-Rad ODS-5S guard column. The solvent gradient was composed of 0.1 M ammonium phosphate (pH 5.8) (solvent A) and HPLC-grade methanol (solvent B) at a flow rate of 1.4 ml/min. The solvent delivery program was as follows: 20% A in B from 0 to 10 min, a linear transition from 20 to 0% A in B from 10 to 18 min, and B only from 18 to 35 min. The injection volume was 50 μl. Commercial standards of Proto IX (Sigma Chemical Co.), Proto IX ME and Mg-Proto IX (Porphyrin Products, Inc., Logan, UT) were used. PChlide was obtained by extraction from etiolated barley (*Hordeum vulgare* L.) tissues, quantified spectrophotometrically as before [34] and injected into the HPLC for calibration of the spectrofluorometric detector. The position of Mg-Proto IX ME was determined by the major peak from extracted DY-treated tissues. This compound causes plants to generate very high levels of Mg-Proto IX ME [35]. Porphyrin detection was performed with fluorescence detector excitation and emission wavelength settings of 400 and 630 nm, respectively, for Proto IX and Proto IX ME; 415 and 595 nm, respectively, for Mg-Proto IX and Mg-Proto IX ME; and 440 and 630 nm, respectively, for PChlide. The photodiode array detector scanned from 300 to 700 nm to confirm all peaks.

In vitro detection of the 503 nm pigment

The assay mixture for Protox assays contained 100 mM HEPES buffer at pH 7.5, 1 mM EDTA, 2 mM DTT, and 7 μM Protogen IX as previously described [20]. The enzyme was preincubated for

20 min in the assay mixture containing DTT before addition of Protogen IX to start the reaction. The enzyme preparations utilized were extracts of barley leaf etioplasts prepared as previously described [33]. In some treatments, enzyme preparations were heated for 15 min at 80 °C. Spectral scans of the reaction mixtures were made with a SLM DW-2000 spectrophotometer in the split beam mode. Scans were made upon addition of Protogen IX and again after incubation at 25 °C for 0.5, 2, 6, and 20 h in complete darkness. All sample handling was under a dim, green light source. The samples were placed in the closest position to the photomultiplier in order to capture as much light as possible from the somewhat turbid membrane preparations (357 mg protein/ml). Spectral scans were stored on a computer so that difference spectra could be generated.

Results

Effect of ALA, AF, and DY on in vivo porphyrin pigments

Only subtle differences were observed between *in vivo* spectra of control, AF-treated, ALA-treated, DY-treated, and ALA plus AF-treated tissues (data not shown) (see Fig. 4 of ref. [23]). However, difference spectra between chemical treatments and controls revealed distinct spectral peaks (Fig. 1), which, when deconvoluted into Gaussian peaks, corresponded approximately with spectral peaks of chlorophyll intermediates obtained by direct measurement of standards in membrane preparations (see Fig. 6 of ref. [23] for an example of the absorption spectrum of Proto IX in a standard membrane preparation), from published data, or measured directly in tissues known to have predominantly one intermediate (Tables I and II). A

Table I. *In vivo* absorbance spectral maxima of chlorophyll intermediates.

Porphyrin	Absorption maxima [nm]	Source of spectral data
Proto IX	411	measurements in membrane
PTP IX	503	ref. [28]
Mg-Proto IX	422, 448, 480	measurements in membrane
Mg-Proto IX ME	422, 450, 483	measurements in membrane
Proto IX ME	411	measurements in membrane
PChlide	446, 633	measurements *in vivo*

Table II. Absorption maxima of *in vivo* difference spectra of chemical treatments minus control tissues and possible corresponding chlorophyll intermediates, as well as compounds detected to be higher than in control tissues by HPLC.

Treatment	Absorption maxima [nm]	Compound Analytical method *In vivo* spectrophotometry	HPLC
AF	409	Proto IX or Proto IX ME	Proto IX and Proto ME
	502	PTP IX?	
ALA	407	Proto IX or Proto IX ME	Proto IX
	633	PChlide	PChlide
	421	Mg-Proto IX or Mg-Proto IX ME	Mg-Proto IX and Mg-Proto IX ME
AF + ALA	409	Proto IX or Proto IX ME	Proto IX and Proto IX ME
	633	PChlide	
	499	PTP IX?	
DY	398	Proto IX or Proto IX ME	Proto IX and Proto IX ME
	421	Mg-Proto IX or Mg-Proto IX ME	Mg-Proto IX and Mg-Proto IX ME
	506	PTP IX?	

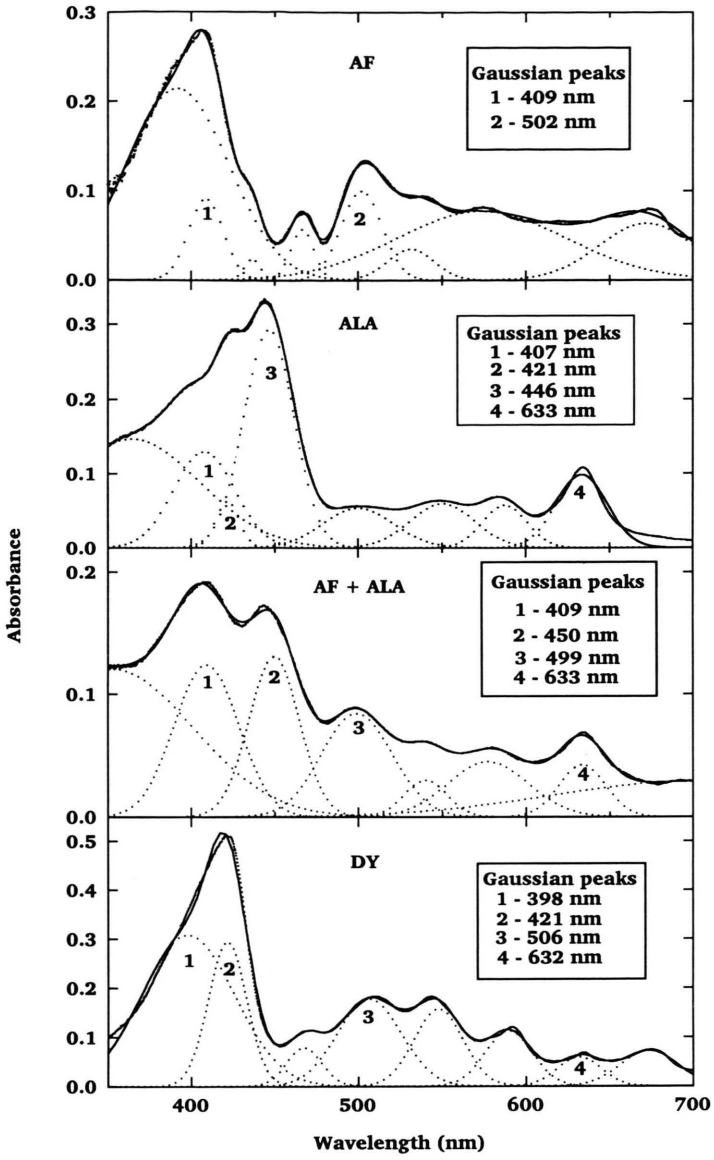

Fig. 1. *In vivo* absorption difference spectra between untreated yellow cucumber cotyledons discs and discs treated with 30 μM AF, 100 μM ALA, 30 μM AF plus 100 μM ALA, or 5 mM DY. Spectra delineated by dots very close together are the actual average measured difference spectra. The spectra delineated by solid lines are made up of the integrated Gaussian curves (separated dots).

peak near 503 nm (499–506 nm) was found only in treatments which also had a large Proto IX peak. This peak was more pronounced in treatments with included AF, but its size varied considerably between experiments. Another example of the AF difference spectra is provided by Duke *et al.* [23]. In this case, a prominent Proto IX peak and a large Gaussian component near 500 nm were also observed.

Deconvolution of the four *in vivo* difference spectra revealed a peak approximating the 411 nm peaks of Proto IX or Proto IX ME in all treatments (Fig. 1). The peaks other than the *ca.* 411 and 503 nm peaks in the AF and the AF plus ALA treatments approximate the minor peaks of Proto IX. Peaks approximating the absorption maxima of Mg-Proto IX or Mg-Proto IX ME (421 nm) were detected in the ALA and DY treatments. Both of the peaks approximating those of PChlide (446 and 633 nm) were in the ALA and ALA plus AF treatments. These qualitative results are summarized in Table II.

HPLC analysis

HPLC analysis of the porphyrins from the treatments revealed pigments that matched those determined by *in vivo* spectrophotometry, except that the 503 nm pigment was not detected (Fig. 2 and 3, Table II). All treatments profoundly increased total porphyrins; however, the patterns and levels of accumulation varied considerably between treatments and porphyrins (Fig. 3).

Attempts to measure the 503 nm pigment with HPLC were unsuccessful. AF plus ALA-treated tissue extracts, Protogen IX-amended control tissue extracts, and Protogen IX samples in methanol were analyzed by HPLC for the presence of this pigment. The entire run was monitored spectrophotometrically at 503 nm. Proto IX could be detected at high levels in the AF plus ALA-treated plant extract and at lower levels in the Protogen IX-amended control plant extract and Protogen IX sample. However, no 503 nm peak was detected in any of the samples.

Attempts to measure the presence of a 503 nm pigment in Protox reaction mixtures

The above *in vivo* spectrophotometric studies suggest the presence of a pigment with an absorption maximum at *ca.* 503 nm in plant leaves treated with AF. To further examine the potential relationship between this 503 nm pigment and the partially oxidized porphyrin intermediate PTP IX, we determined whether a compound with a 503 nm absorption maximum could be detected in Protox

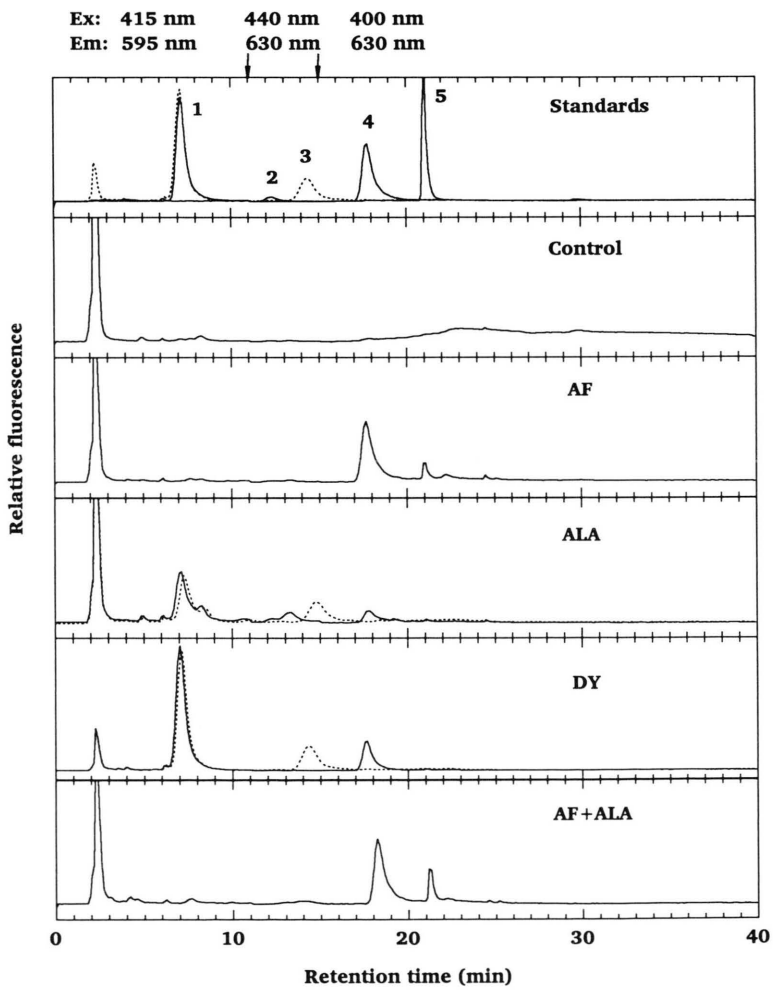

Fig. 2. HPLC profiles of extracts of porphyrin standards and treatments discussed in text. Excitation and emission wavelengths during the chromatographic run are indicated at the top of the graph, except for dotted lines, in which case the profile was produced with continuous 415 nm excitation and 595 nm emission. The standards are as follows: 1, Mg-Proto IX; 2, PChlide; 4, Proto IX; and 5, Proto IX ME. All standards were injected at concentration of 1 μM and the *y*-axis range is −0.005 to 0.070. Compound 3 (dotted line) is Mg-Proto IX ME caused to accumulate in cucumber tissue treated with 5 mM DY for 20 h in darkness (*y*-axis range is −0.09 to 0.70).

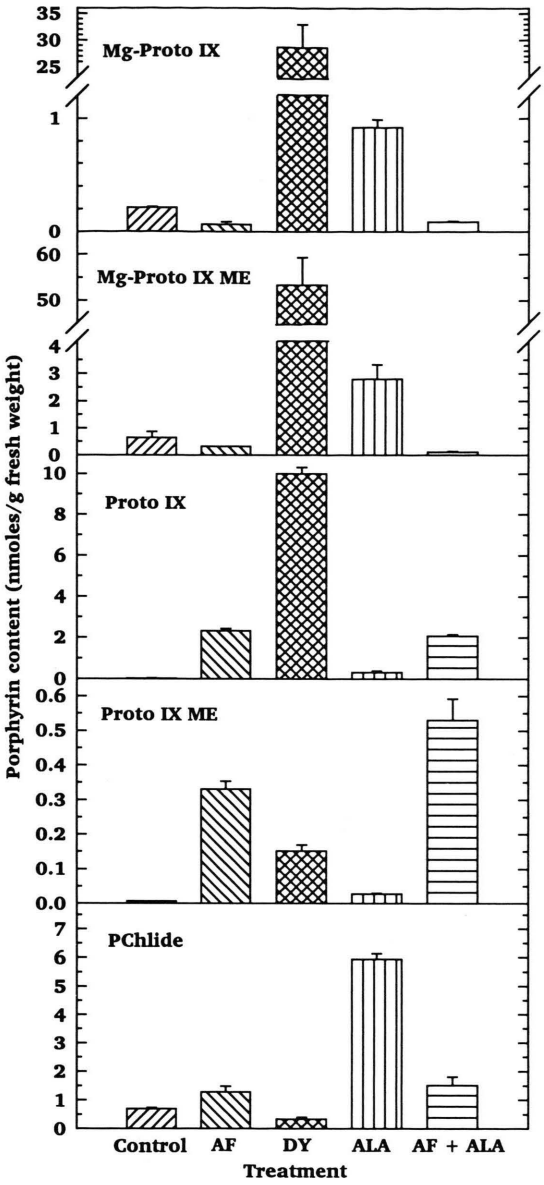

Fig. 3. Porphyrins of treatments of Fig. 1 and 2 as determined by HPLC. Error bars are one standard error of the mean.

reaction mixtures incubated with AFM, the methylated form of AF which is a significantly better Protox inhibitor than AF [1, 6].

Barley etioplasts were the enzyme source. We have previously shown that extracts of barley etioplasts have easily measured Protox activity that can be inhibited by AFM [6]. Protogen IX was incubated in an extract of barley etioplasts, both in the presence or absence of AFM. The experiment was repeated with different amounts of DTT in the reaction mixture. After incubation for 0.5, 2, 6, and 20 h, visible spectra of the reaction mixtures were scanned spectrophotometrically.

With 2 mM DTT, after 2 h of the enzymatic incubation without AFM, the difference spectra between the spectra at this time and at 0 h had spectral peaks characteristic of membrane-associated Proto IX (see Table I) only (Fig. 4A). The same difference spectrum (2 h minus 0 h) with a heated preparation resulted in no dominant absorption peaks (Fig. 4A). In the presence of AFM and Pro-

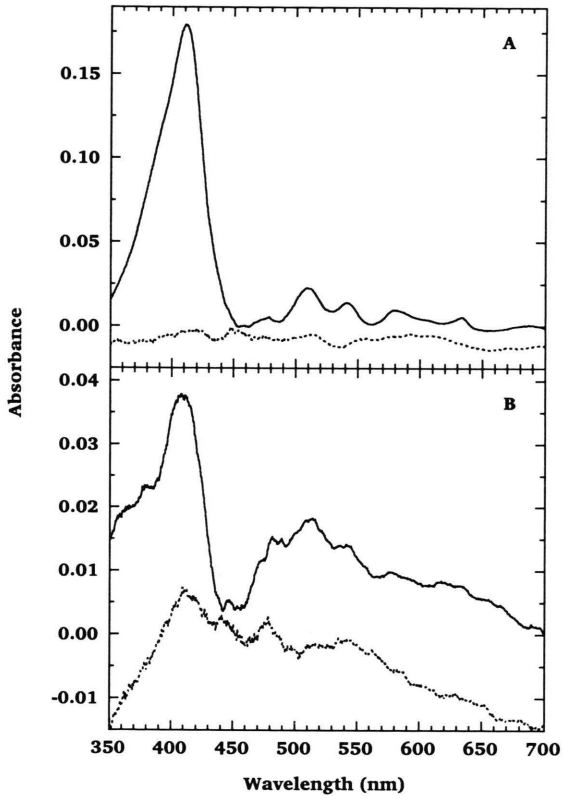

Fig. 4. Effect of AFM on the presence of a 503 nm pigment in Protox preparations incubated with Protogen IX. Barley etioplast extracts (357 mg protein/ml) were incubated with Protogen IX in a 1 ml assay mixture (described in Materials and Methods) in the absence (A) or presence of 1 μM AFM (B). Spectral scans are difference spectra of unheated 2 h incubation minus 0 h incubation (solid curves of A and B) and the difference spectra of heated 2 h incubation minus 0 h incubation (dotted curves of A and B).

togen IX, the Proto IX peak was much smaller and a secondary peak near 503 nm was sometimes much larger, relative to the Proto IX peak (Fig. 4B). This secondary peak is similar to that reported for the partially oxidized PTP IX form of Protogen IX [27, 29]. The difference spectrum of a heated preparation with AFM yielded a peak near that of Proto IX with no other dominant absorption maximum (Fig. 4B). The Proto IX peak in the uninhibited reaction was lower and the Proto IX and 503 nm peaks in the inhibited reaction were lower or missing at 0.5, 6, and 20 h than at 2 h (data not shown). Furthermore, with different DTT concentrations, the 503 nm peak was absent or not as pronounced. The optimal time for detection should be a function of rates of production and destruction and the oxidizing environment. These factors may change with time. Protox-generated Proto IX was associated with purified etioplast (by Percoll gradient) and plasma membrane preparations (by two-phase partition [36]); however, no 503 nm peak was found in these assays (data not shown).

Discussion

The porphyrins indicated by the *in vivo* difference spectra (Fig. 1, Table II) are identical to those previously identified by HPLC in green cucumber cotyledons discs exposed to the same treatments [34], except for the 503 nm pigment. The *in vivo* spectrophotometric results also agreed with the HPLC results from the same treatments except for the 503 nm pigment (Table II).

The 503 nm pigment could not be detected by our HPLC method for several possible reasons. If it is PTP IX, it might be oxidized to Proto IX or other products during sample preparation. Our extraction, HPLC, or detection methods could be insufficient for proper analysis. This is likely to be the case if it is not PTP IX.

The results of Fig. 4 suggest that the 503 nm pigment is present *in vitro* in Protox assays in the presence of AFM under some conditions. In contrast, enzymatic Protogen IX oxidation in the absence of AFM yields primarily Proto IX, with very little or no accumulation of the 503 nm compound. An unexpected result was that no 503 nm peak occurred in heated samples, but did occur in the unheated reaction with AFM. This suggests that the AFM-resistant, Protox-like activity associated with plant membranes [20] may be required for formation of the 503 nm pigment. Furthermore, it suggests that this activity is enzymatic. An alternative explanation is that the 503 nm peak is due to enzymatic production of a non-porphyrin compound with a 503 nm peak, as suggested by Labbe-Bois and Labbe [37] for the *in vivo* 503 nm peak in yeast. However, we think that this is highly unlikely in our system, because the peak did not occur without the presence of Protogen IX *in vitro* or high Proto IX levels *in vivo*. Furthermore, it is unlikely that a full complement of enzyme and substrate for the production of a non-porphyrin pigment would be associated with isolated membranes. Nevertheless, our inability to detect the 503 nm pigment by HPLC and the limited conditions under which it was detectable *in vitro* preclude us from identifying it as PTP IX.

Tetrahydroporphyrins have been characterized chemically as relatively stable intermediates in the chemical autooxidation of porphyrinogens (hexahydroporphyrins) to fully oxidized porphyrins [34], and PTP IX can be characterized by its 503 nm absorption maximum [27, 28, 37]. A pigment with a 503 nm absorption maximum similar to that of PTP IX has been observed in the cytoplasmic fraction of yeast growing anaerobically or in glucose minimal media [27, 38] and in *E. coli* [39]. Evidence indicates that the appearance of the 503 nm pigment is associated with growth conditions leading to decreased Protox activities both in an *E. coli* mutant [39] and in a yeast [29]. These findings have suggested that when rapid enzymatic Protox activity is decreased or stopped, PTP IX accumulates as a result of a slower non-Protox-mediated oxidation of Protogen IX. Our finding of a 503 nm pigment in plants treated with a Protox-inhibiting herbicide suggests a similar conclusion.

Proto IX ME (Fig. 2 and 3) has not been previously reported to accumulate in tissues treated with Protox-inhibiting herbicides. Whether methylation of Proto IX under these circumstances occurs in and/or outside of the plastid is not known. However, if Protox-inhibiting herbicides cause Proto IX to accumulate primarily outside the plastid [19, 20], it is likely that the Proto IX ME is formed outside the plastid. This seems even more likely in that this compound was found at a lower concentration in DY-treated than in AF-treated

tissues, even though the DY-treated tissues had sixfold higher levels of Proto IX (Fig. 3). We assume that Proto IX accumulating in DY-treated tissues accumulates primarily in the plastid.

In vivo spectrophotometry is not quantitative because of light scattering, compartmentation, and other factors [23]. Therefore, the absolute or relative amounts of different pigments in a tissue cannot be determined accurately by *in vivo* spectrophotometry. However, our results demonstrate that this method is a rapid, reliable method to determine if a herbicide is porphyrinogenic. Furthermore, the pattern of accumulation can indicate the enzyme target site in the porphyrin pathway.

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