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Investigation of the subcellular location of the tetrapyrrole-biosynthesis enzyme coproporphyrinogen oxidase in higher plants

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The subcellular location of two enzymes in the biosynthetic pathway for protoporphyrin IX, coproporphyrinogen (coprogen) oxidase (EC 1.3.3.3) and protoporphyrinogen (protogen) oxidase (EC 1.3.3.4) has been investigated in etiolated pea (*Pisum sativum*) leaves and spadices of cuckoo-pint (*Arum maculatum*). Plant tissue homogenized in isotonic buffer was subjected to subcellular fractionation to prepare mitochondria and plastids essentially free of contamination by other cellular organelles, as determined by marker enzymes. Protogen oxidase activity measured fluorimetrically was reproducibly found in both mitochondria and etioplasts. In contrast, coprogen oxidase could be detected only in etioplasts, using either a coupled fluorimetric assay or a sensitive radiochemical method. The implications of these results for the synthesis of mitochondrial haem in plants is discussed.

INTRODUCTION

The pathway of tetrapyrrole biosynthesis leads to the formation of many end products in photosynthetic organisms, including chlorophyll, haem, phytochromobilin and sirohaem. The first committed precursor is 5-aminolaevulinic acid (ALA) which is made from the intact carbon skeleton of glutamate via the C_5 pathway (Kannangara et al., 1988) and is converted by a series of reactions into protoporphyrin IX, the last common intermediate between haem and chlorophyll. Insertion of Fe^{2+} into protoporphyrin IX produces protohaem, whereas chelation of Mg^{2+} is the first step on the chlorophyll branch.

Isolated chloroplasts readily synthesize chlorophyll from [^{14}C]ALA or [^{14}C]glutamate (Fuesler et al., 1984; Gomez-Silva et al., 1985), indicating that the entire pathway for the production of chlorophyll is present in plastids. Ferrochelatase activity is also found in etioplasts and mature chloroplasts (Jones, 1968; Little and Jones, 1976) and so they can presumably also make protohaem. However, haem is found elsewhere in the cell, most notably in the mitochondria, but also in haemoproteins of the endoplasmic reticulum and microbodies. In animals, ALA is made in mitochondria from succinyl-CoA and glycine in a single reaction catalysed by the enzyme ALA synthase. The ALA then leaves the mitochondrion and is converted into coproporphyrinogen III (coprogen) in the cytosol. This then re-enters the mitochondrion, where the last three enzymes to protohaem are located [for a review, see Dailey (1990)]. The same situation prevails in yeast, except that coprogen oxidase is cytosolic (Camadro et al., 1986). Initially it was assumed to be true also for the synthesis of mitochondrial haem in plants (in other words there was, in addition to the chloroplast pathway, another one shared between mitochondrion and cytosol), but recent work suggests that this is not the case. There is no convincing evidence for the presence of ALA synthase in plants, and it is now thought that all cellular ALA is made via the C_5 pathway in the plastids (e.g. Castelfranco and Jones, 1975; Schneegurt and Beale, 1986; Werck-Reichhardt et al., 1988). Similarly, the next two enzymes of the pathway, ALA dehydratase and porphobilinogen (PBG) deaminase, which are cytosolic in animals and yeast, have been shown by subcellular-fractionation techniques to be confined to the plastid, both in pea (*Pisum sativum*) leaves, and in spadices of *Arum* (Smith, 1988). This latter tissue is non-photosynthetic and undergoes a considerable increase in mitochondrial activity prior to thermogenesis (ap Rees et al., 1983). Thus any tetrapyrrole-synthesizing capacity is likely to be for mitochondrial haem rather than for chlorophyll.

In contrast, the last two enzymes of haem synthesis, ferrochelatase and protoporphyrinogen (protogen) oxidase, have been detected both in mitochondria and purified plastids from several plants (Little and Jones, 1976; Jacobs and Jacobs, 1987; Matringe et al., 1989a,b), although the extent of contamination of the mitochondrial fraction by chloroplast protogen oxidase was not addressed directly. Since the last two steps of mitochondrial haem synthesis takes place in mitochondria, some earlier intermediate must pass from the plastid to mitochondrion because, in plants, the reactions that lead to the formation of hydroxymethylbilane are located exclusively in the former organelle. At present, the location of the other enzymes of the pathway remains unproven, although it is likely that, given the instability of hydroxymethylbilane, uroporphyrinogen III synthase is in the same compartment as PBG deaminase (i.e. the plastid). The only report of uroporphyrinogen III decarboxylase in plants found it principally in the soluble fraction of crude tobacco (*Nicotiana tabacum*) leaf homogenates (Chen and Miller, 1974), but no attempt was made to estimate the extent of organelle breakage. Since probably more than 60% of plastids are routinely broken by most homogenization procedures, these data cannot be taken to mean that the enzyme is cytosolic, but simply soluble. A similar criticism can be levelled at the report on coprogen oxidase activity in tobacco (Hsu and Miller, 1970). Although the authors conclude that it was associated primarily with the mitochondria, these were prepared simply by differential centrifugation, and indeed are described as containing broken chloroplasts.

In order to provide more information on the identity of the tetrapyrrole intermediate which passes from the plastid to the

Abbreviations used: ALA, 5-aminolaevulinic acid; PBG, porphobilinogen; NAD$^+$-ME, NAD$^+$ malic enzyme; PPase, alkaline pyrophosphatase; 6PGDH, 6-phosphogluconate dehydrogenase; AdK, adenylate kinase.

mitochondrion, we have investigated more thoroughly the subcellular location of both coprogen oxidase and protogen oxidase in plant tissue. The implications of the data for the tetrapyrrole pathway in plants are discussed.

MATERIALS AND METHODS

Materials

Biochemicals were from Sigma, Poole, Dorset, U.K., or Boehringer-Mannheim, Lewes, East Sussex, U.K. Coproporphyrin III hydrochloride and protoporphyrin IX hydrochloride were obtained from Porphyrin Products, Logan, UT, U.S.A. Yeast mitochondrial membranes prepared as described by Labbe et al. (1985) were a gift from Dr. P. Labbe, Institut Jacques Monod, Paris, France. [$^{14}C_2$]Coproporphyrin III, ^{14}C-labelled in the carboxy carbon atoms of the 2- and 4-propionic acid substituents, was synthesized as described by Elder and Evans (1978a).

Plant material

Peas (*Pisum sativum*, var. Feltham First) were sown in compost and grown in complete darkness at 22 °C for 7 days. Spadices from cuckoo-pint (*Arum maculatum* L.) at the β-stage (ap Rees et al., 1983) were collected locally.

Subcellular fractionation

Total homogenate of etiolated pea leaves or *Arum* spadices was prepared by chopping tissue with a razor blade in isotonic medium (Smith, 1988). Subsequent fractionation on sucrose gradients to prepare mitochondria and etioplasts was also essentially as described by Smith (1988), except that gradients of 35 ml were used. These were centrifuged at 25000 rev./min in a Beckman SW27 rotor for 15 min for pea and 3 h for *Arum*, and were fractionated into 17 samples of 2 ml each, together with the gradient pellet.

Enzyme assays

NAD$^+$ malic enzyme (NAD$^+$-ME) activity was measured as described by ap Rees et al. (1983), alkaline pyrophosphatase (PPase) as described by Gross and ap Rees (1986), 6-phosphogluconate dehydrogenase (6PGDH) as described by Bergmeyer et al. (1983) and adenylate kinase (AdK) by monitoring the amount of ATP formed as described by Brolin (1983).

Coprogen oxidase was measured fluorimetrically in a coupled assay exactly as described by Labbe et al. (1985), except that the fluorescence was measured on a Perkin–Elmer MPF 44B spectrofluorimeter. This method measures the increase in protoporphyrin IX fluorescence with time in a reaction mixture containing tissue extract under investigation, coprogen III and excess protogen oxidase (provided by yeast mitochondrial membranes, which are essentially devoid of endogenous coprogen oxidase activity), to convert the protogen IX into protoporphyrin IX. This is detected fluorimetrically and can be distinguished from oxidized coproporphyrin because they have different emission-wavelength ($\lambda_{em.}$) maxima (Labbe et al., 1985; Smith and Griffiths, 1993). EDTA is included to inhibit any chelatase activity which might utilize the protoporphyrin IX generated, and chemical oxidation of the coprogen III is minimized by the addition of high concentrations of reducing agent. The reactions were carried out at 30 °C in a total volume of 3 ml and contained, in addition to tissue extract (5–250 μg of protein), 100 mM potassium phosphate, pH 7.6, saturated with air, 1 mM EDTA, 6 mM dithiothreitol, 2 mg of Tween 80, 50 μl of yeast mitochondrial membranes (30 mg of protein/ml) and 6 μM coprogen III (prepared from coproporphyrin III by chemical reduction with sodium amalgam). The excitation wavelength ($\lambda_{ex.}$) was 410 nm, and $\lambda_{em.}$ was 632 nm with slit widths of 5 nm. Protogen oxidase was measured in an identical assay, except that protogen IX was substituted for coprogen III, and no yeast membranes were added. For both enzymes, assays were carried out with at least two different protein concentrations, and non-enzymic blanks of equivalent amounts of extract boiled for 10 min were always included to determine the rate of chemical oxidation of the porphyrinogen.

Coprogen oxidase activity was also measured by radiochemical determination of released CO_2 (Elder and Evans, 1978a). Reactions were carried out for 1 h at 37 °C with shaking in a total volume of 100 μl and contained 75 mM potassium phosphate buffer, pH 7.2, 10 mg/ml BSA, 1 mM KOH, 18–20 μM [$^{14}C_2$]-coprogen III (1.7 mCi/mmol) and up to 50 μl of tissue extract (5–50 μg of protein). $^{14}CO_2$ was collected in 1.0 M Hyamine solution in methanol, and radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter. Under these conditions, the minimum detection limit of the assay was about 50 pmol of CO_2/h.

RESULTS

Activity of tetrapyrrole biosynthesis enzymes in etiolated pea leaves

Table 1 shows the results of using the two fluorimetric assays to determine coprogen oxidase and protogen oxidase activities in crude extracts of etiolated leaves of pea seedlings grown in the dark for 7 days. For comparison, the rates of two other tetrapyrrole biosynthesis enzymes, ALA dehydratase and PBG deaminase (Smith, 1988), are also given. For the assay of coprogen oxidase, it was found essential to ensure that the fluorescence observed was indeed due to protoporphyrin IX ($\lambda_{em.}$ 632 nm) rather than coproporphyrin III ($\lambda_{em.}$ 610 nm),

Table 1 Activity of tetrapyrrole-biosynthesis enzymes in etiolated pea leaves

Pea leaves were chopped in isotonic buffer, filtered through muslin to remove debris and the activity of enzymes determined in the homogenate. Coprogen and protogen oxidase activities were measured spectrofluorimetrically as described in the Materials and methods section, and expressed as nmol of protoporphyrin IX/h. ALA dehydratase activity (expressed as nmol of PBG/h) and PBG deaminase activity (nmol of uroporphyrin/h) are taken from Smith (1988). Values are expressed as means ± S.D. Because four PBG molecules are required for each tetrapyrrole molecule, the ALA dehydratase rate must be divided by 4 to be compared with the others.

	Activity	
Enzyme	(nmol/h per mg of protein)	(nmol/h per g fresh wt.)
ALA dehydratase*	30.33 ± 5.15	177.60 ± 31.18
PBG deaminase*	1.59 ± 0.19	9.30 ± 1.13
Coprogen oxidase†	0.41 ± 0.136	2.35 ± 0.61
Protogen oxidase‡	3.81 ± 0.91	17.36 ± 0.36

* Smith (1988).
† Mean of four measurements.
‡ Mean of six measurements.

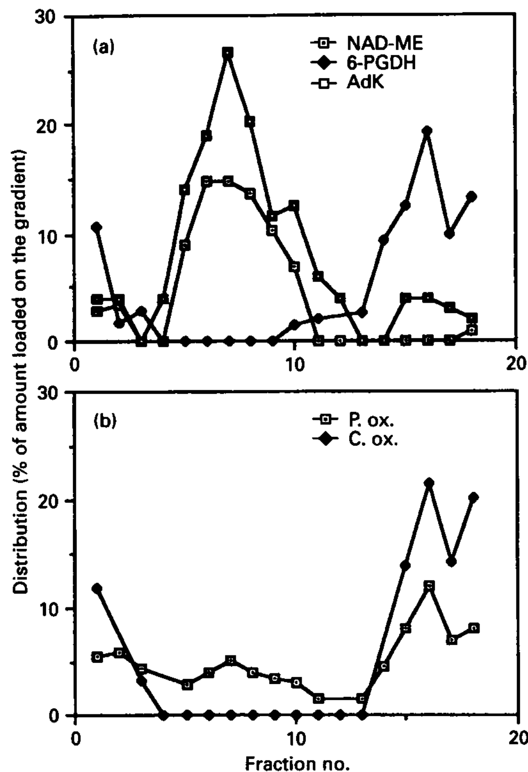


Figure 1 Distribution of enzyme activities in sucrose gradients of etiolated pea leaf homogenates

(**a**) Distribution of marker enzymes in gradient, expressed as a percentage of activity loaded on gradient. ▣, NAD$^+$-ME (mitochondrial matrix); ◆, 6PGDH (plastid); ■, AdK (mitochondrial intermembrane space). (**b**) Distribution of (◆) coprogen oxidase (C. ox.) and (▣) protogen oxidase (P. ox.) activities in the same gradient fractions, expressed as a percentage of the activity loaded on to the gradient. Fraction 1 is the top of the gradient; fraction 18 is the gradient pellet.

which arises non-enzymically by chemical oxidation of the substrate coprogen III. In contrast, high background rates were not observed with crude organelle preparations (i.e. 10000 *g* pellets; results not shown), suggesting that a soluble component(s) of the total leaf lysate was interfering with enzyme determination.

It is possible, therefore, that the rate obtained fluorimetrically for coprogen oxidase in this etiolated tissue is an underestimate. It is slightly lower than that found in homogenates of fully green tobacco leaves (Hsu and Miller, 1970). There is no report of the activity of protogen oxidase in total homogenates, but values of 1–20 nmol/h per mg of protein have been reported for organelles from different plants (Jacobs and Jacobs, 1987; Matringe et al., 1989a,b; Camadro et al., 1991), comparable with that found here. The rate of chlorophyll synthesis during the early stages (6–12 h) of greening of etiolated pea leaves is of the order of 3 nmol/h per g fresh weight (Smith, 1986), and so it would appear that coprogen oxidase levels may be limiting (but see below). However, the activity of both ALA dehydratase and PBG deaminase increase during greening (Smith, 1986; A. Wallace-Cook and A. G. Smith, unpublished work), and it is conceivable that, after 6 h illumination, there would be higher levels of coprogen oxidase.

Subcellular fractionation of etiolated pea leaf tissue

The total homogenates of etiolated pea leaf tissue were fractionated into mitochondria and etioplasts using the method developed previously (Smith, 1988). The homogenates were centrifuged at 10000 *g* to produce a crude organellar preparation, which was then fractionated further on a 35–55 % (w/v) sucrose gradient with a 65 % sucrose cushion. After centrifugation, two bands were visible in the gradient: a diffuse white band half way through and a yellow one at the interface with the 65 % sucrose cushion. At the bottom of the gradient was a starchy pellet. The gradients were separated into 2 ml fractions, and each was assayed for marker enzymes and coprogen and protogen oxidase activities. The results of a representative experiment are presented in Figure 1. The top panel shows the distribution of marker enzymes NAD$^+$-ME (for mitochondria; ap Rees et al., 1983) and 6PGDH, which is found in both the cytosol and plastids (Schnarrenberger et al., 1973). The distribution of PPase, which is exclusively plastidic (Gross and ap Rees, 1986) paralleled that of 6PGDH in gradients (results not shown). From these results it is clear that the upper white band corresponds to the mitochondria, while the band at the interface is the etioplast fraction. The first two fractions contain the broken organelles and cytosol which did not enter the gradient, while the gradient pellet is comprised of amyloplasts and of incompletely resuspended material. Also shown on the Figure is the distribution of activity of AdK, which is located within the intermembrane space of the mitochondrion (Stitt et al., 1982). Since it parallels the distribution of NAD$^+$-ME, the mitochondria clearly have their outer membrane intact.

In Figure 1(b) the activities of coprogen oxidase and protogen oxidase (both determined fluorimetrically) in each fraction are

Table 2 Distribution of enzymes activities after subcellular fractionation of homogenate of etiolated pea leaves

A homogenate of pea leaves was centrifuged at 10000 *g* and the resuspended pellet loaded on to a sucrose gradient as described in the Materials and methods section. Gradients were fractionated into 17 × 2 ml fractions + gradient pellet and each fraction was assayed for marker enzymes and coprogen and protogen oxidase, the latter two fluorimetrically. The total activity recovered in fractions corresponding to the mitochondria and etioplasts are given, expressed as percentages of the amount in the 10000 *g* pellet loaded on the gradient.

Fraction	NAD$^+$-ME	PPase	6PGDH	AdK	Coprogen oxidase	Protogen oxidase
Activity loaded on gradient (μmol/h)	33.3	43.7	23.88	6.64	4.39×10^{-3}	8.47×10^{-3}
Activity recovered in mitochondrial fraction (%)	52.0	3.1	N.D.*	62.7	N.D.*	15.0
Activity recovered in etioplast fraction (%)	1.0	45.3	51.4	2.1	54.6	31.6
Total recovery (%)	76.8	78.0	86.0	113.5	84.8	80.8

* N.D., not detectable.

Table 3 Subcellular localization of coprogen oxidase using the radioactive assay method

Homogenates of pea leaves and *Arum* spacides were centrifuged at 10000 *g*, and the resuspended pellets were loaded on to sucrose gradients as described in the Materials and methods section. Gradients were fractionated into 17 × 2 ml fractions + gradient pellet and each fraction assayed for marker enzymes and coprogen oxidase using the radioactive method of Elder and Evans (1978a, b). The total activity recovered in fractions corresponding to the broken organelles at the top of the gradient, mitochondria and etioplasts are given, expressed as percentages of the amount in the 10000 *g* pellet loaded on the gradient. It is not possible to prepare intact plastids from *Arum* using this gradient system.

Fraction	Pea leaves				*Arum* spadices			
	NAD^+-ME	PPase	AdK	Coprogen oxidase*	NAD^+-ME	PPase	AdK	Coprogen oxidase*
Activity loaded on gradient (μmol/h)	8.21	27.89	3.02	42.7×10^{-3}	28.08	1.20	1.09	7.13×10^{-3}
Activity recovered at top of gradient (broken organelles) (%)	6.1	15.9	3.6	17.0	12.5	92.3	11.0	87.9
Activity recovered in mitochondrial fraction (%)	51.1	7.0	48.8	N.D.†	58.0	N.D.†	55.3	N.D.†
Activity recovered in etioplast fraction (%)	N.D.†	67.0	5.7	65.9	–	–	–	–
Total recovery (%)	79.0	94.2	115.5	103.9	118.0	92.3	89.5	94.8

* In nmol of $^{14}CO_2$ released/h.
† N.D., not detectable.

presented. The distribution of the former enzyme closely resembles that of the plastids in the gradient, and no activity was detected in the mitochondrial fraction. In contrast, while protogen oxidase activity is found in the etioplast fraction, it is also easily measurable in the mitochondria. The same trend is evident from the data in Table 2, which gives the distribution of enzyme activity in the different fractions from the gradient as a percentage of that loaded on the gradient. Protogen oxidase is consistently present in the mitochondrial fraction at levels much greater than that of contaminating plastids, as indicated by the recovery of 6PGDH and PPase in this fraction. On average the relative proportions of protogen oxidase found in etioplasts and mitochondria were about 2:1, although this varied between experiments, most probably because of the differing extents of organelle breakage.

Although these fractionation studies were carried out several times, on no occasion was coprogen oxidase activity demonstrated convincingly in the mitochondrial fraction, even when the amount of extract added to the 3 ml assay exceeded 250 μg of protein, while as little as 5 μg of etioplast protein was sufficient to obtain a measurable rate. Similarly protogen oxidase activity could be detected with 15 μg of mitochondrial protein. Nevertheless, it is possible that the conditions used to measure the plastid coprogen oxidase may not be appropriate for a putative mitochondrial isoenzyme, and so a number of parameters were altered, including varying the concentration of substrate (from 1 to 15 μM), substituting Triton X-100 for Tween 80, and using buffers at different pH values (7.0–8.0); again, no activity was convincingly observed (results not shown).

Radioactive coprogen oxidase assay

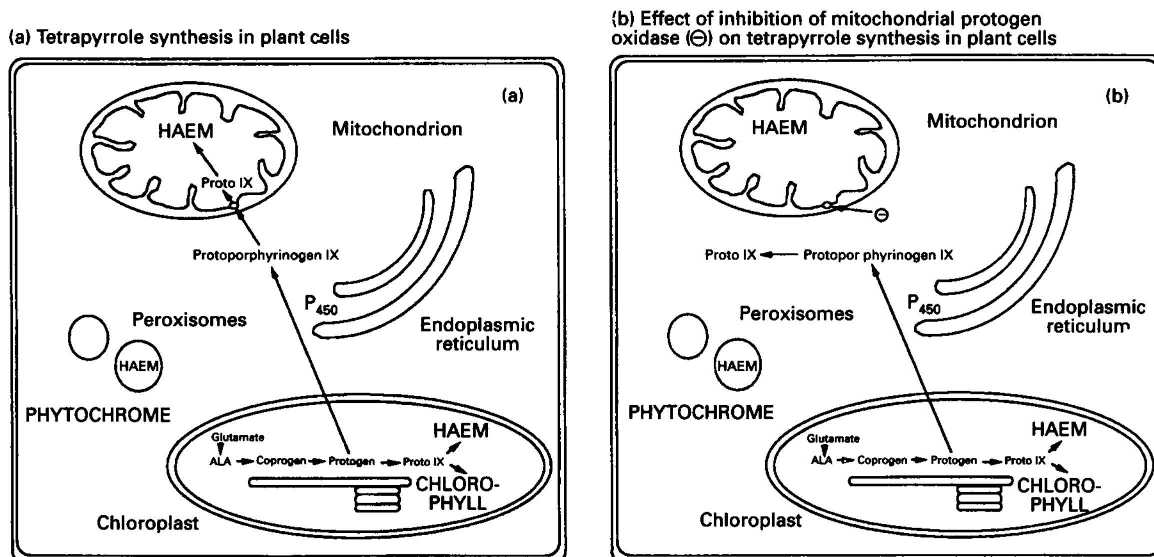
Although the results in the previous section suggest that there is no coprogen oxidase in pea leaf mitochondria, if it is present at very low levels (mitochondrial haem constituting only about 1 % of cellular tetrapyrrole in green leaf) it is possible that the coupled assay method, with detection limits of 0.1 nmol/h per 3 ml of incubation mixture, is not sufficiently sensitive to detect it. To try to address this criticism, an alternative assay method was employed in which the rate of reaction is monitored by measuring the amount of $^{14}CO_2$ released from the substrate coprogen labelled with ^{14}C in the carboxy atoms of the 2- and 4-propionate groups (Elder and Evans, 1978a). In addition, mitochondria from another plant tissue, spadices of *Arum maculatum* were also assayed. This tissue undergoes a massive increase in mitochondrial activity just prior to thermogenesis (ap Rees et al., 1983), so any tetrapyrrole synthesis capacity would be expected to be for mitochondria rather than plastids, and a putative mitochondrial isoenzyme would be expected to be more easily detected.

The activity of coprogen oxidase in total homogenates of etiolated pea leaf and *Arum* spadices measured by the release of $^{14}CO_2$ was found to be 7.3 and 2.52 nmol/h/mg of protein respectively (45.7 and 21.9 nmol/h per g fresh wt.). Since two molecules of CO_2 are released per molecule of coprogen III oxidized, the values must be halved if they are to be compared with those measured by the fluorimetric assay. This means that the levels of coprogen oxidase measured radiochemically are comparable with the activity of protogen oxidase in etiolated pea leaves (Table 1), and would be more than sufficient to account for the observed rate of greening observed between 6 and 12 h of illumination. An explanation for the different sensitivity of the two assays for coprogen oxidase may be that the radiochemical method is not subject to high background rates observed with crude extracts in the fluorimetric procedure.

When the tissue homogenates are fractionated on sucrose gradients as described above and assayed (Table 3), once again coprogen oxidase activity is readily detectable in pea leaf etioplasts (it is not possible to isolate intact plastids from *Arum* using this gradient system). However, none was observed in mitochondria from either peas or *Arum*. Although some radioactivity was measured in mitochondrial fractions, these were less than twice those obtained in blanks without enzyme and did not increase with increasing protein concentration.

DISCUSSION

The results presented here demonstrate for the first time unequivocally that the tetrapyrrole-synthesis enzyme protogen oxidase is present in plant mitochondria which are essentially free of plastids (Figure 1b and Table 2). Although there have been previous reports of protogen oxidase activity in mitochondria from various plants (Jacobs and Jacobs, 1987; Matringe et al., 1989a,b), they did not rule out the possibility of con-



Scheme 1 Distribution of tetrapyrrole-synthesis intermediates in plant cells

(**a**) Schematic model of a typical plant cell showing distribution of cellular tetrapyrroles and intermediates in their synthesis. (**b**) Schematic model showing the potential effects of prevention of protogen IX from entering the mitochondrial matrix, for instance by inhibition of mitochondrial protogen oxidase. Abbreviations: Proto IX, protoporphyrin X; P_{450}, cytochrome *P*-450.

tamination by plastids. However, we have been unable to demonstrate the presence of the preceding enzyme coprogen oxidase in plant mitochondria using either a coupled fluorimetric assay or a radiochemical method (with detection limits as low as 50 pmol of CO_2/h). In contrast, both methods detect the enzyme in purified etioplasts (Figure 1b and Tables 2 and 3). In animals, coprogen oxidase is located in the intermembrane space of mitochondria (Elder and Evans, 1978b; Grandchamp et al., 1978), and so most activity is lost if the outer membrane ruptures. However, this is not the explanation for the absence of activity in the plant mitochondrial preparations used here, since these were shown to possess adenylate kinase activity, a marker for the intermembrane space (Stitt et al., 1982). Although our data appear to be contradictory to the work of Hsu and Miller (1970), who concluded that coprogen oxidase was preferentially associated with the mitochondrial fraction of tobacco leaves, in fact their actual data are compatible with the present paper, since they describe their mitochondrial preparation as containing broken chloroplasts.

While we are confident that coprogen oxidase is not present in plant mitochondria, we cannot rule out the possibility of an isoform of coprogen oxidase in the cytosol, where it is found in yeast (Camadro et al., 1986). With the technique used here of aqueous fractionation of organelles, it is virtually impossible to avoid breakage of plastids. Thus any cytosolic preparation would be contaminated with soluble enzymes from these organelles, and it would be very difficult to distinguish a plastidic enzyme from a cytosolic form present at low levels. Recently, however, a cDNA for coprogen oxidase has been isolated from soybean (*Glycine max*) (O. Madsen, N. Sandals and K. Marcker, unpublished work). It appears to be a single-copy gene, and the cDNA encodes a polypeptide which is imported into isolated pea chloroplasts and processed to a smaller form (M. A. Santana and A. G. Smith, unpublished work). This is consistent with the view that there is only one form of the enzyme and it is plastidic, although differential processing of transcripts from a single gene is not excluded by this evidence.

Nevertheless, our results strongly suggest that protogen IX must be transported into the mitochondrion from the cytosol to act as substrate for protogen oxidase. A model for the distribution of tetrapyrrole intermediates in plant cells is shown in Scheme 1a. A possible consequence of the transport of protogen IX from the plastid to the mitochondrion is that, if its uptake into mitochondria is prevented in some way, it may accumulate in the cytosol, where some or all is likely to oxidize non-enzymically to protoporphyrin IX, a process which occurs spontaneously in the presence of light and oxygen. If the protoporphyrin IX cannot pass into the mitochondria or plastids, it cannot be utilized as a substrate by the chelatase enzymes which are considered to be within the organelles, and so would be a metabolic dead end (Scheme 1b). If this model is correct, it would provide an explanation for the effects of diphenyl ether herbicides, such as acifluorofen-methyl, which specifically inhibit protogen oxidase (Matringe et al., 1989a,b; Witowski and Halling, 1989). Surprisingly, it is protoporphyrin IX (its product) which accumulates. This is sequestered in cell membranes and causes photo-oxidative damage in the light. Interestingly, an equivalent situation prevails in human variegate porphyria, where protogen oxidase activity is deficient, but once again protoporphyrin IX accumulates (Brenner and Bloomer, 1980; Deybach et al., 1981). Presumably the protogen IX produced in the intermembrane space by coprogen oxidase can cross the mitochondrial outer membrane to the cytosol, but not the inner membrane into the matrix, towards which the active site of ferrochelatase is orientated (Jones and Jones, 1969; Harbin and Dailey, 1985).

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