Measurement of ferrochelatase activity using a novel assay suggests that plastids are the major site of haem biosynthesis in both photosynthetic and non-photosynthetic cells of pea (*Pisum sativum* L.)

Johanna E. CORNAH[1], Jennifer M. ROPER, Davinder Pal SINGH[2] and Alison G. SMITH[3]
Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, U.K.

Ferrochelatase is the terminal enzyme of haem biosynthesis, catalysing the insertion of ferrous iron into the macrocycle of protoporphyrin IX, the last common intermediate of haem and chlorophyll synthesis. Its activity has been reported in both plastids and mitochondria of higher plants, but the relative amounts of the enzyme in the two organelles are unknown. Ferrochelatase is difficult to assay since ferrous iron requires strict anaerobic conditions to prevent oxidation, and in photosynthetic tissues chlorophyll interferes with the quantification of the product. Accordingly, we developed a sensitive fluorimetric assay for ferrochelatase that employs Co^{2+} and deuteroporphyrin in place of the natural substrates, and measures the decrease in deuteroporphyrin fluorescence. A hexane-extraction step to remove chlorophyll is included for green tissue. The assay is linear over a range of chloroplast protein concentrations, with an average specific activity of 0.68 nmol · min^{-1} · mg of $protein^{-1}$, the highest yet reported. The corresponding value for mitochondria is 0.19 nmol · min^{-1} · mg of $protein^{-1}$. The enzyme is inhibited by *N*-methylprotoporphyrin, with an estimated IC_{50} value of ≈ 1 nM. Using this assay we have quantified ferrochelatase activity in plastids and mitochondria from green pea leaves, etiolated pea leaves and pea roots to determine the relative amounts in the two organelles. We found that, in all three tissues, greater than 90 % of the activity was associated with plastids, but ferrochelatase was reproducibly detected in mitochondria, at levels greater than the contaminating plastid marker enzyme, and was latent. Our results indicate that plastids are the major site of haem biosynthesis in higher plant cells, but that mitochondria also have the capacity for haem production.

Key words: ferrochelatase assay, purified organelle, recovery of marker enzyme.

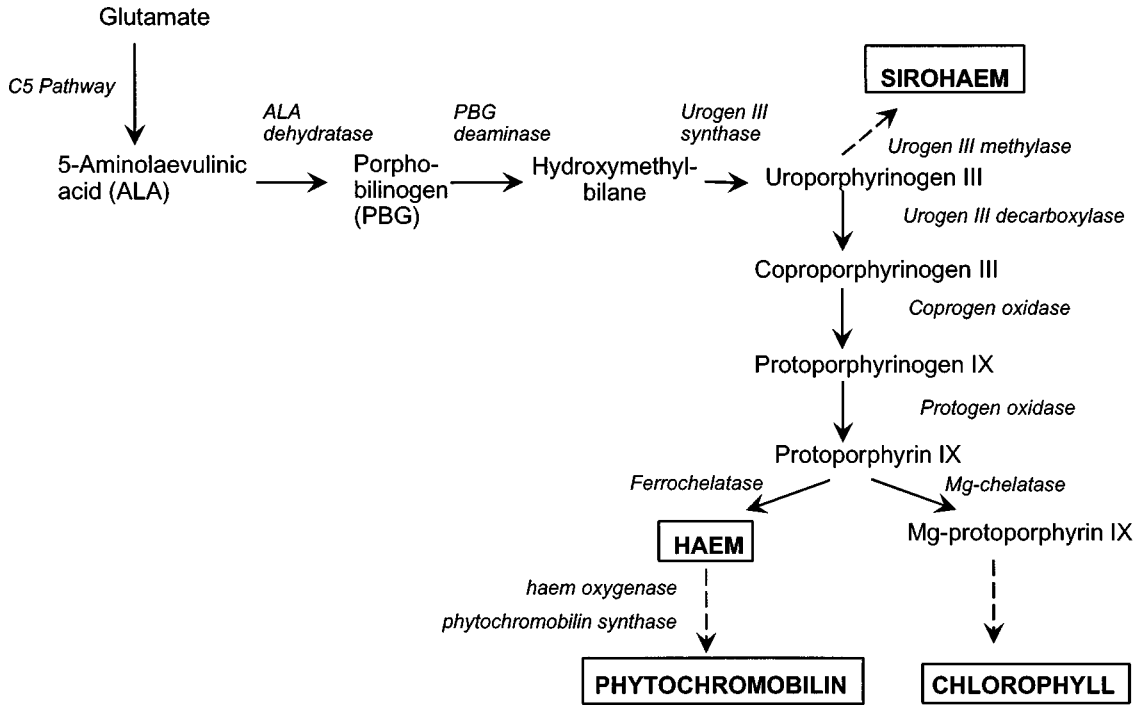
INTRODUCTION

Haem and chlorophyll are synthesized in a common branched pathway in plants and algae [1]. Although chlorophyll is far more abundant, haem has a fundamental role in a wide range of plant metabolic reactions, including electron transfer, oxidation reactions and oxygen homoeostasis. The terminal enzyme of haem biosynthesis is ferrochelatase, which catalyses the insertion of ferrous iron into protoporphyrin IX, the last common intermediate of haem and chlorophyll synthesis (Scheme 1). Ferrochelatase is thus at the branchpoint between the two pathways. Jones [2] first detected its activity in higher plants in cell-free extracts, and it was detected and partially characterized in spinach leaves [3,4]. Activity was found associated with the mitochondrial fraction of potato tubers and the chloroplast and etioplast fractions of oat and bean seedling leaves [4]. This work was extended by Little and Jones [5], who used marker enzymes to quantify the purity of organelle fractions; they were able to measure ferrochelatase activity in both plastids and mitochondria from etiolated barley seedlings. Subsequently, ferrochelatase activity was reported in plasma membranes from barley seedlings, in addition to mitochondria and etioplasts [6]. Given the fact that haemoproteins are found throughout the plant cell, it is possible that haem is made *in situ*, for assembly with its cognate apoproteins. Further support for this possibility comes from the fact that activity of the preceding enzyme of the pathway, protoporphyrinogen IX oxidase (protogen oxidase), is found in both plastids and mitochondria [7–9], and a cDNA for a mitochondrial isoform of protogen oxidase was isolated from tobacco (*Nicotiana tabacum*) [10]. Protogen oxidase activity has also been measured in plasma membrane [11,12] and endoplasmic reticulum [13], although this activity had different characteristics from those in the plastids and mitochondria. In contrast, all the steps up to and including coproporphyrinogen III oxidase are confined to plastids [9,14–16], and it is likely that protogen IX is exported from these organelles to other subcellular locations. Evidence for the export of this compound was obtained after the incubation of isolated barley plastids with the tetrapyrrole precursor 5-aminolaevulinic acid [17].

We have isolated two cDNAs for ferrochelatase from *Arabidopsis thaliana* (*AtFC-I* and *AtFC-II*), by functional complementation of a yeast *hem15* mutant [18,19]. There are no other genes for ferrochelatase in the recently completed *Arabidopsis* genome [20]. The proteins encoded by these cDNAs share 69 % identity at the amino acid level, and both have N-terminal extensions characteristic of transit peptides. Despite the similarity of the amino acid sequences, it is likely that two ferrochelatase isoforms have different roles within the cell. The *AtFC-II* gene was expressed only in aerial tissues [19], whereas that for *AtFC-I* was expressed ubiquitously [18]. Promoter analysis of the genes (D. P. Singh, J. E. Cornah and A. G. Smith, unpublished work) suggests that AtFC-II is important for the synthesis of haem for photosynthetic cytochromes, whereas AtFC-I is involved in the synthesis of haem for other subcellular compartments. In cu-

Abbreviations used: ALAD, 5-aminolaevulinic acid dehydratase; NAD-ME, NAD-malic enzyme; protogen oxidase, protoporphyrinogen IX oxidase.

[1] Present address: Institute of Cell and Molecular Biology, University of Edinburgh, Kings Buildings, Mayfield Road, Edinburgh EH9 3JR, U.K.
[2] Present address: CSIRO-Plant Industry, Horticultural Unit, Merbein, Victoria 3505, Australia.
[3] To whom correspondence should be addressed (e-mail alison.smith@plantsci.cam.ac.uk).



Scheme 1 Biosynthetic pathway of tetrapyrroles in plant cells, showing the major endproducts (boxed)

The C5 pathway comprises three enzymes: Glu-tRNA synthetase, Glu-tRNA reductase and glutamate-1-semialdehyde (GSA) aminotransferase. The dashed lines represent several enzymic steps.

cumber (*Cucumis sativus*), genes for two ferrochelatase isoforms have been identified (*CsFeC1* and *CsFeC2*) that are equivalent to the two *Arabidopsis* enzymes. These too have differing tissue expression patterns: transcripts for CsFeC1 were found in hypocotyl, roots and flowers, but not in cotyledons or mature leaves, whereas those for CsFeC2 were found in all tissues and were light-responsive [21,22]. In tobacco (*Nicotiana tabacum*), only one ferrochelatase gene has been identified to date, which belongs to the same class as *AtFC-II* and *CsFeC2* [23]. Transgenic tobacco plants containing antisense constructs for this gene (*NtFeCh*) accumulate high levels of protoporphyrin IX and display a severe necrotic phenotype, indicating that if there is another ferrochelatase present in tobacco, it cannot substitute for NtFeCh.

The type II ferrochelatase precursors from *Arabidopsis*, cucumber and tobacco were imported *in vitro* into chloroplasts, but not mitochondria [19,22,23]. In contrast, the precursor proteins of AtFC-I and CsFeC1 were imported into both isolated pea chloroplasts and mitochondria [22,24], as was barley type I ferrochelatase (D. P. Singh and A. G. Smith, unpublished work). However, import into pea mitochondria has been shown not to be specific since these organelles will import several bona fide chloroplast precursors, including plastocyanin and the small subunit of Rubisco [25,26]. Furthermore, mitochondria purified from *Arabidopsis* cell-suspension cultures did not import the AtFC-I precursor [25], leading the authors to propose that plant mitochondria do not contain ferrochelatase. This contradicts the widespread detection and analysis of ferrochelatase in plant mitochondria (e.g. [4,5,23]), but it remains possible that the activity measured is due to contamination of the mitochondria with plastids.

To address this issue directly, we have taken a quantitative biochemical approach to determine the relative levels of ferrochelatase within plastids and mitochondria. Because ferrochelatase is a difficult enzyme to assay *in vitro*, particularly in photosynthetic plant tissues where chlorophyll interferes with the accurate quantification of product, we have developed a novel assay. It is based on one devised by Brown et al. [27] to measure the enzyme in the red alga *Cyanidium caldarium*, and is both reproducible and sensitive. Using this method, we have assayed ferrochelatase in both plastids and mitochondria from green leaves, etiolated leaves and roots from pea (*Pisum sativum* L.), and quantified the contribution of each organelle to overall ferrochelatase activity, and thereby haem synthesis, within the plant cell.

EXPERIMENTAL

Growth of plants

Seeds of *P. sativum* L. var. Feltham First and *A. thaliana*, ecotype Lansdberg erecta, were surface-sterilized with 10% bleach, imbibed for 3–5 h and planted on sterilized potting compost. Plants were grown for 7–12 days in a greenhouse at an ambient temperature of 15–25 °C with artificial lighting to provide 16 h of constant illumination followed by 8 h of darkness. Alternatively, pea plants were germinated and grown in the dark at an ambient temperature of 15–25 °C to produce etiolated seedlings. If roots were required, the peas were germinated and grown hydroponically in distilled water in the dark. Cauliflowers, tulips and chrysanthemums were obtained from a local market.

Isolation of organelles from green pea leaves

Mitochondria and chloroplasts were isolated from 10–12-day-old pea leaves and grown under a light/dark cycle, as above, using Percoll gradients as described by Chow et al. [24], except

that the final washes for the mitochondrial preparation were with resuspension medium without EDTA.

Isolation of organelles on a single sucrose gradient

Mitochondria and plastids were isolated from etiolated leaves and root tissue as described by Smith [14]. After centrifugation the gradients were fractionated into 2 ml fractions and the plastid and mitochondrial fractions collected. The organelles were then washed in 50 mM Hepes/NaOH (pH 7.6)/0.33 M sorbitol and centrifuged at 15000 ***g*** for 15 min at 4 °C.

Preparation of crude organelle pellets

Plant tissue (5–10 g) was chopped with a razor blade in a minimal volume of ice-cold chopping buffer [50 mM Hepes/KOH (pH 7.8)/0.33 M sorbitol/1 mM $MgCl_2$/0.0015% dithiothreitol/10% (w/v) polyvinylpolypyrrolidone] until homogeneous. The homogenate was filtered through a single layer of miracloth and centrifuged at 12000 ***g*** for 12 min at 4 °C. The crude organelle pellet was resusupended in a minimal volume of chopping buffer and kept on ice in the dark.

Protein and chlorophyll determination

Protein concentration was determined using the BioRad protein assay kit using the manufacturer's protocol, with BSA as a standard. Chlorophyll was measured in acetone-extracted samples as described in [28].

Preparation of porphyrin solutions

Porphyrins were obtained from Porphyrin Products (Logan, UT, U.S.A.). Deuteroporphyrin IX and protoporphyrin IX were obtained as the dihydrochloride salts. A stock solution of ≈ 1 mM was prepared in 0.5 M Tris/HCl, pH 7.8, containing 0.15% Tween 80 solution (for deuteroporphyrin) and 2% Tween 80 (for protoporphyrin IX). The concentrations of the stocks were determined using the absorption coefficients for deuteroporphyrin ($\epsilon_{mM} = 433\ mM^{-1} \cdot cm^{-1}$ at 398 nm) and protoporphyrin ($\epsilon_{mM} = 262\ mM^{-1} \cdot cm^{-1}$ at 405 nm) [29]. The monomeric nature of the porphyrins in aqueous solution was monitored regularly by scanning 10 μM stock solutions in a spectrophotometer. A monomeric solution has four characteristic peaks between 450 and 650 nm. Protoporphyrinogen IX was made by reduction of protoporphyrin IX with sodium amalgam as described in [30].

Ferrochelatase assay: 'Co-deuteroporphyrin assay'

The optimized assay (see the Results section) was carried out as follows: a 5 × assay mix [75 μM deuteroporphyrin IX/0.5 M Tris/HCl (pH 7.6 for mitochondria or 7.8 for plastids)/0.15% Tween 80/150 μM $CoSO_4$] was prepared just prior to use. All organelle and plant preparations were diluted to 2 mg of protein · ml^{-1} with lysis buffer (10 mM Tris/HCl, pH 7.6 or 7.8) and placed on ice to lyse for 5 min. The final 0.5 ml assay contained 100 μl of 5 × mix, 150 μl of water and 250 μl of plant extract, which was added to start the reaction. The tubes were incubated in the dark at 26 °C. At fixed time points, three 20 μl aliquots were taken from the assay tube and added to 80 μl of acetone to stop the reaction, followed by centrifugation to remove precipitated protein. Each sample was made up to 500 μl with 80% (v/v) acetone. For chloroplast samples, the chlorophyll was removed by adding 500 μl of hexane. After vortexing, the sample was centrifuged at 10000 ***g*** for 2 min to separate the phases, and the hexane layer was removed. Recovery experiments showed that 90% of the deuteroporphyrin IX was recovered in the acetone fraction, irrespective of the initial porphyrin concentration. To determine the deuteroporphyrin IX content, samples were diluted six times with 80% (v/v) acetone in a quartz cuvette (four polished faces) and the fluorescence measured in a PerkinElmer LS 50 luminescence spectrophotometer. An excitation wavelength of 400 nm was used and the fluorescence intensity recorded at 620 nm. Excitation and emission slits were 5 and 10 nm respectively. Known concentrations of deuteroporphyrin IX dissolved in 80% (v/v) acetone were used to calibrate the fluorimeter.

Other enzyme assays

5-Aminolaevulinic acid dehydratase (ALAD), a plastid-specific marker enzyme [14], and NAD-malic enzyme (NAD-ME), a mitochondrion-specific marker enzyme [31], were assayed in total homogenate and in all pellet and supernatant fractions from each organelle-isolation procedure, using the methods described in [14]. Protogen oxidase activity was determined by the continuous fluorimetric method described in [30].

RESULTS

Co-deuteroporphyrin assay for ferrochelatase activity

Several assays have been described for ferrochelatase using the endogenous substrates protoporphyrin IX and ferrous iron, including determination of ^{59}Fe incorporation into haem [3], the formation of pyridine haemochromogens from haematoporphyrins [4] and the spectrofluorimetric and spectrophotometric disappearance of porphyrin substrates in continuous assays [4,32,33]. However, these assays are technically complex since they require totally anaerobic conditions to maintain ferrous iron in the reduced state, and to overcome these difficulties alternative methods have been developed. The most widely used is that described for the yeast enzyme, which is a continuous spectrophotometric assay measuring the rate of formation of zinc-protoporphyrin IX [34]. However, zinc is not a good substrate for higher-plant ferrochelatase [5]. In our hands, although we were able to measure some zinc-protoporphyrin IX production with isolated pea chloroplasts, the rates obtained were not reproducible, and we were unable to detect any activity with isolated pea mitochondria using this assay method (results not shown). In order for us to embark on a quantitative comparison of ferrochelatase activity in different higher-plant organelles, it was necessary to develop an alternative procedure.

To this end, an assay developed for the red alga *C. caldarium* [27] gave promising results. This stopped assay, described in detail in the Experimental section, uses cobalt and deuteroporphyrin IX as substrates. The advantage of using Co^{2+} is that it remains bivalent under aerobic conditions. Deuteroporphyrin IX lacks the vinyl substituents at positions 3 and 8 of the porphyrin macrocycle found in protoporphyrin IX, and is much more soluble and less likely to dimerize in aqueous solution than protoporphyrin IX [35]. It has been widely used in assays of ferrochelatase (e.g. [5,21,36]), and has been shown to be a better substrate for plant ferrochelatase than protoporphyrin IX *in vitro* [5]. Because the product Co-deuteroporphyrin does not fluoresce, the disappearance of the substrate deuteroporphyrin is measured over time.

However, the assay required modification to improve its sensitivity for use with higher-plant extracts, since *C. caldarium* has very high endogenous ferrochelatase activity to provide haem for the synthesis of the bilin chromophore of the photo-

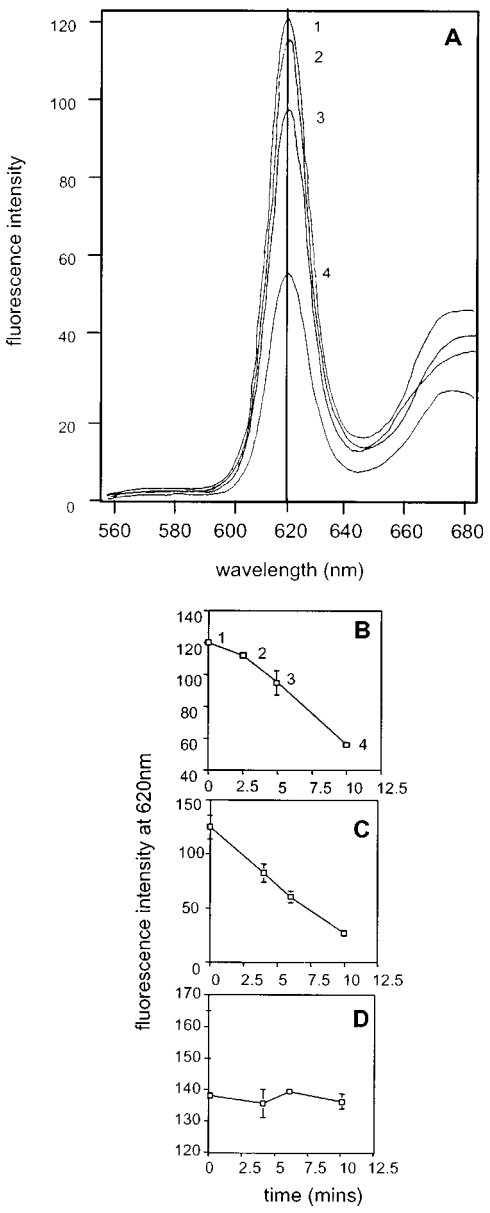


Figure 1 Measurement of ferrochelatase activity with the Co-deuteroporphyrin assay

Lysed pea chloroplasts (1 mg · ml^{-1}) were incubated with 15 μM deuteroporphyrin and 30 μM $CoSO_4$ as described in the Experimental section. Fluorescence was measured with an excitation wavelength of 400 nm in an LS 50 luminescence spectrometer, using slit widths of 5 nm (excitation) and 10 nm (emission). (**A**) Fluorescence spectra of samples taken at 0 min (curve 1) and at 2 (curve 2), 4 (curve 3) and 10 (curve 4) min after addition of plant extract to the assay mix. The peak at 680 nm represents residual chlorophyll fluorescence. (**B**) Fluorescence intensity at 620 nm from results in (**A**) plotted against time. (**C**) Rate of change of fluorescence intensity at 620 nm in an assay carried out after preincubation of assay mix and plant extract at 26 °C. (**D**) Rate of change of fluorescence intensity at 620 nm in an assay carried out with boiled plant extract.

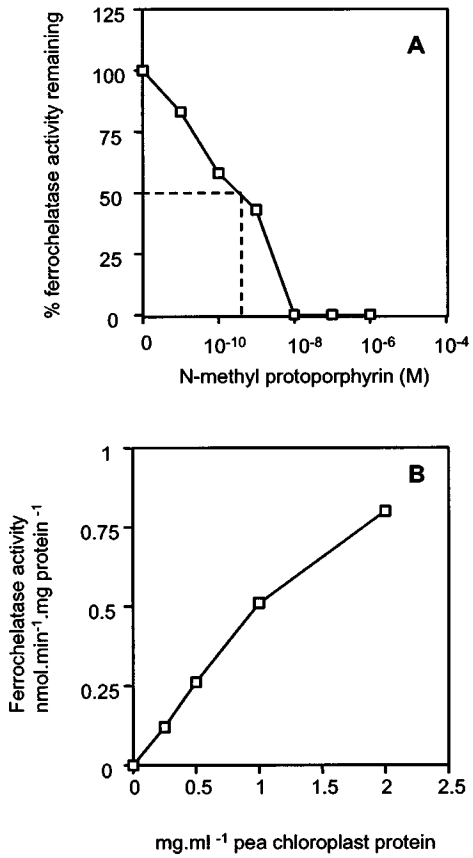


Figure 2 Characteristics of ferrochelatase activity in isolated pea chloroplasts measured with the Co-deuteroporphyrin assay

(**A**) Effect of *N*-methylprotoporphyrin on incorporation of Co^{2+} into deuteroporphyrin IX. Ferrochelatase activity is expressed as a percentage of the remaining activity. Values are means from three separate experiments. (**B**) Effect of chloroplast protein concentration on incorporation of Co^{2+} into deuteroporphyrin IX. Values are from a single representative experiment, and are means from two separate assays.

synthetic antenna protein, phycocyanin. We therefore introduced a hexane-extraction step to remove chlorophyll, and in addition we used a fluorimeter to detect deuteroporphyrin IX, rather than a spectrophotometer. Figure 1(A) shows the results of a typical assay using pea chloroplasts. Despite some residual chlorophyll fluorescence at about 680 nm, the deuteroporphyrin IX peak at 620 nm was clear, and this decreased over the 10 min time course of the assay. The rate is plotted against time in Figure 1(B) and, after an initial lag, was essentially linear. The lag was eliminated if both the plant sample and the reaction mix were pre-warmed to 26 °C before mixing together in the assay (Figure 1C). No decrease in deuteroporphyrin fluorescence was seen in control assays with boiled chloroplasts (Figure 1D), or with no plant extract (results not shown).

In order to verify that this assay was specific for ferrochelatase, we tested the effect of a specific inhibitor, *N*-methylprotoporphyrin [37], on the enzyme activity (Figure 2A). There is a dramatic reduction in ferrochelatase activity, with an apparent IC_{50} of $\approx$ 1 nM. In addition, we investigated the effect of altering the protein concentration, and found that it was essentially linear up to 1 mg · ml^{-1} (Figure 2B). The levelling off at higher protein concentrations may have been due to the difficulty in removing all the chlorophyll, which then led to increased interference with the determination of deuteroporphyrin IX fluorescence.

The mean ferrochelatase specific activity in pea chloroplasts measured with this Co-deuteroporphyrin assay was 0.68 nmol · min^{-1} · mg of protein^{-1}. This is some 10-fold higher than rates that we could determine with the zinc-protoporphyrin assay in [33], and two to three times higher than previously published values, which were between 0.15 and 0.36 nmol · min^{-1} · mg of protein^{-1}

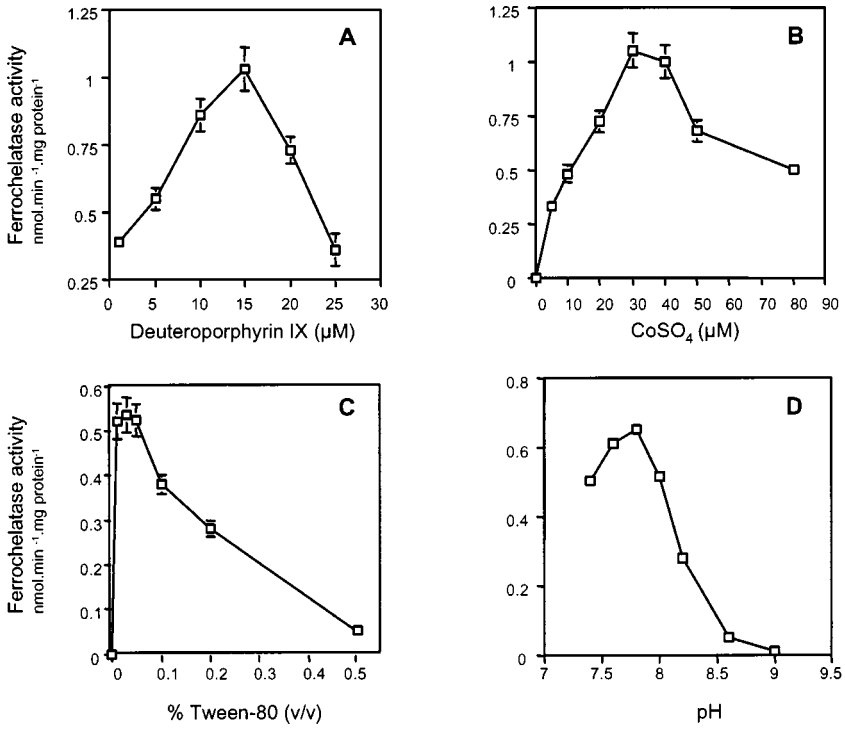


Figure 3 Optimization of the substrate concentrations and assay conditions for pea chloroplast ferrochelatase

Assays were set up as described in Figure 1, and fluorescence measurements of triplicate samples were taken at each time point to enable the rates of the disappearance of deuteroporphyrin IX fluorescence to be calculated. The effect of altering the following assay conditions was determined: (**A**) deuteroporphyrin IX concentration, (**B**) $CoSO_4$ concentration, (**C**) Tween 80 concentration and (**D**) pH (with Tris/HCl buffer). The graphs show the means ± S.E.M. from three assays at each concentration/pH.

[4,36]. Using the Co-deuteroporphyrin assay, we measured the specific activity of ferrochelatase in crude cell lysates of *Escherichia coli* and *Saccharomyces cerevisiae*, and obtained values of 0.305 and 0.85 nmol · min^{-1} · mg of protein^{-1}, respectively.

Optimization of the assay

In order to optimize the conditions for the assay with plant tissue, a number of parameters were tested. Pea chloroplasts, at a protein concentration of 1 mg · ml^{-1}, were used throughout these optimizations as they contain high, readily detectable levels of ferrochelatase activity. Optimal substrate concentrations were found to be 15 μM deuteroporphyrin IX and 30 μM $CoSO_4$ (Figures 3A and 3B). The drop in ferrochelatase activity at concentrations of deuteroporphyin IX above 15 μM (Figure 3A) was unexpected, and suggested initially that the enzyme was experiencing substrate inhibition. However, it is in fact likely to be due to increasing amounts of the detergent Tween 80, which was included in the assay to maintain deuteroporphyrin's solubility. Detergent has been reported to inhibit ferrochelatase activity [3]. We therefore examined the effect of Tween 80 concentration on our assay, and found that activity decreased sharply above 0.05 % Tween 80 (Figure 3C), with the optimum being 0.03 %. Accordingly, all subsequent experiments contained 0.03 % Tween 80, whatever the deuteroporphyrin IX concentration. As a result, the sharp decrease in activity above 15 μM deuteroporphyrin IX was no longer observed (results not shown), although this remained the optimal concentration. The optimal pH for chloroplast lysate was 7.8 (Figure 3D), with a rapid decrease in activity seen above pH 8.

Kinetic parameters of ferrochelatase in chloroplasts

Having optimized the assay, the kinetic parameters of ferrochelatase in pea chloroplasts were investigated. The substrate versus velocity curve for deuteroporphyrin IX (Figure 4A) indicated that the kinetics did not fit the Michaelis–Menten model, since the curve was not hyperbolic and showed little plateauing at high substrate concentration. Nevertheless, an estimate for the V_{max} of 1.6 nmol · min^{-1} · mg of protein^{-1} and a K_m of 5 μM could be made from the Hanes–Woolf plot (Figure 4B). This is similar to the K_m of 2.4 μM reported for deuteroporphyrin IX with pea chloroplasts [36]. Varying the Co^{2+} concentration produced hyperbolic kinetics (results not shown), with an estimated K_m of 1.25 μM. For comparison, the K_m values for ferrochelatase in *C. caldarium* were reported to be 6.5 and 14.8 μM for deuteroporphyrin IX and Co^{2+}, respectively [27]. Assuming the situation in pea is the same as in *Arabidopsis* and cucumber, where two ferrochelatase isoforms are targeted to chloroplasts [19,22], kinetic studies of ferrochelatase activity within chloroplasts will be complicated, especially if the two isoforms have different reaction profiles. Ideally, both isoforms need to be characterized independently after separation, as has been done for purified recombinant ferrochelatase-1 from cucumber [21]. In this case, K_m values of 14.4 and 4.7 μM were determined for deuteroporphyrin IX and Fe^{2+}, respectively.

As ferrochelatase is a branchpoint enzyme, regulation of its activity *in vivo* is essential to control the relative flux through the haem and chlorophyll branches. Little and Jones [5] studied the effect of metalloporphyrins on ferrochelatase in barley etioplasts and mitochondria, and found that they inhibited activity. When Fe-protoporphyrin (protohaem) and Mg-protoporphyrin were

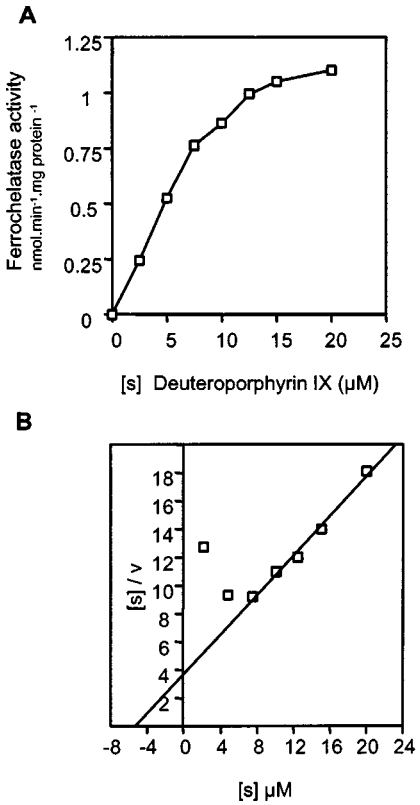


Figure 4 Kinetic parameters of pea chloroplast ferrochelatase

(**A**) Rate of ferrochelatase activity measured using the Co-deuteroporphyrin assay with varying deuteroporphyrin IX concentration. (**B**) Hanes–Woolf plot of the same data. V_{max} was estimated to be 1.6 nmol · min^{-1} · mg of $protein^{-1}$ and K_m was 5.0 μM. Data are from a representative experiment using triplicate assays, in which the errors were less than 0.02 nmol · min^{-1} · mg of $protein^{-1}$.

Table 1 Effect of different compounds on ferrochelatase activity in pea chloroplasts

Ferrochelatase activity was determined, using the Co-deuteroporphyrin stopped assay, in isolated pea chloroplasts (at a protein concentration of 1 mg · ml^{-1}), using the standard assay conditions of 15 μM deuteroporphyrin, following the addition of the compounds listed. The values are means from two independent experiments. n.d., not detectable; proto, protoporphyrin IX.

Assay conditions	Ferrochelatase activity (nmol · min^{-1} · mg of $protein^{-1}$)	Activity remaining (%)
30 μM Co^{2+} (control)	0.67	100
30 μM Co^{2+} + 1 μM Fe-proto	0.32	48
30 μM Co^{2+} + 10 μM Fe-proto	n.d.	0
30 μM Co^{2+} + 1 μM Mg-proto	0.47	70
30 μM Co^{2+} + 10 μM Mg-proto	0.16	24
30 μM Co^{2+} + 1 μM ATP	0.33	49
30 μM Co^{2+} + 10 μM ATP	0.26	39

included in the Co-deuteroporphyrin assay with pea chloroplasts, both compounds inhibited activity, with complete abolition of activity in the presence of 10 μM protohaem (Table 1). Another means of regulating ferrochelatase might be via adenine nucleotides, since Mg-chelatase requires ATP for activity [38]. Furthermore, haem synthesis in isolated pea chloroplasts was shown to be inhibited, and Mg-protoporphyrin synthesis enhanced, in the presence of ATP [39]. Addition of ATP to pea chloroplasts reduced incorporation of Co^{2+} into deuteroporphyrin IX, with about 39 % activity remaining at 10 μM ATP (Table 1).

Table 2 Ferrochelatase activity in different tissues of higher plants

After homogenization of plant material in isotonic buffer followed by filtration to remove debris, the homogenate was centrifuged at 12000 ***g*** for 12 min to obtain a crude organelle preparation. Ferrochelatase activity was then measured using the Co-deuteroporphyrin assay, at a protein concentration of 1 mg · ml^{-1}.

Plant	Tissue	Ferrochelatase activity (nmol · min^{-1} · mg of $protein^{-1}$)
Pea	Leaf	0.11
	Flower	0.178
	Root	0.121
Arabidopsis	Leaf	0.09
Cauliflower	Leaf	0.035
	Flower	0.105
Tulip	Leaf	0.055
	Flower	0.185
Chrysanthemum	Leaf	0.095
	Flower	0.21

Ferrochelatase activity in pea mitochondria and in crude organellar preparations

In order to assay ferrochelatase activity in pea mitochondria, similar optimizations were carried out with respect to substrate concentration and pH, using mitochondria from pea at a protein concentration of 1 mg · ml^{-1}. The optimum concentrations of cobalt and deuteroporphyrin IX were the same as for the chloroplast assay, suggesting similar K_m values, but the pH optimum was 7.6 rather than 7.8 (results not shown). The specific activity of ferrochelatase was about 0.2 nmol · min^{-1} · mg of $protein^{-1}$. Furthermore, *N*-methylprotoporphyrin inhibited the enzyme, with 53 % inhibition seen at 1 nM.

The assay was also sensitive enough to measure ferrochelatase activity in crude organelle preparations from several higher plant tissues, although not from total homogenates. In addition, we were unable to detect the enzyme reproducibly in tobacco, even in partially purified organelle preparations. This is probably because of the high levels of interfering phenolic compounds present in this tissue, since addition of tobacco leaf homogenate to pea chloroplasts caused a marked decrease in detectable ferrochelatase activity (results not shown).

Nevertheless, we were able to determine ferrochelatase activity in different tissues of several higher plants (Table 2). In all cases, ferrochelatase activity was higher in flowers than in leaves or roots. This is an interesting result, since analysis of the expression pattern of the two *Arabidopsis* ferrochelatase genes using promoter–reporter gene fusions indicates that there is higher expression of both AtFC-I and AtFC-II in flowers than in leaves (D. P. Singh, J. E. Cornah and A. G. Smith, unpublished work).

Activity of ferrochelatase in chloroplast and mitochondria

The main aim of our work was to investigate the extent to which haem biosynthesis is carried out in different organelles. To this end, ferrochelatase activity was assayed in purified plastids and mitochondria isolated from green leaves, etiolated leaves and roots of peas (Table 3). The specific activity of ferrochelatase was markedly higher in chloroplasts from green leaves (10–12-day-old plants) than in plastids from etiolated leaves or roots (7–8-

Table 3 Ferrochelatase specific activity in pea organelles

Ferrochelatase activity in purified pea organelles assayed at 1 mg of protein · ml^{-1} using the Co-deuteroporphyrin assay. Values are means ± S.E.M., $n \geqslant 4$.

	Ferrochelatase activity (nmol · min^{-1} · mg of protein^{-1})	
Tissue	Plastids	Mitochondria
Green leaves	0.678 ± 0.22	0.191 ± 0.12
Etiolated leaves	0.184 ± 0.07	0.216 ± 0.01
Roots	0.209 ± 0.03	0.124 ± 0.02

day-old plants). The activity in mitochondria was similar in both leaf tissues, but somewhat lower in root mitochondria. In green leaves the activity in chloroplasts was over three times higher than in mitochondria (Student's t test, $P < 0.005$), whereas in etiolated leaves, plastids and mitochondria have a similar level of activity ($P = 0.42$). Ferrochelatase activity in roots was significantly higher in plastids than in mitochondria ($P = 0.026$).

However, the specific activity measurements do not provide any indication of the relative contributions of ferrochelatase activity in chloroplasts and mitochondria to total ferrochelatase activity in the cell, since during the purification of the organelles there is considerable loss of material. Furthermore, the total protein content of the two organelles is quite different, so the values are simply a measure of the abundance of the enzyme relative to the other proteins in each organelle. We therefore determined the recovery of each organelle in the purified preparations, using as marker enzymes ALAD for plastids [14] and NAD-ME for mitochondria [31]. The results of a representative experiment for each tissue are presented in Table 4. For green leaves, 9.8 % of the chloroplasts and 28 % of the mitochondria were recovered in our final preparation. Assuming that the recovery of ferrochelatase is the same as for the marker enzymes, the activity of ferrochelatase measured in each organelle preparation can be used to calculate the amount this represents in the total homogenate, and values of 185 and 0.543 nmol · min^{-1} were obtained. Thus, within a unit mass of tissue, the proportion of ferrochelatase in the mitochondria is only 0.3 % of the total, although this is nevertheless greater than the level of contaminating plastid marker enzyme. A similar pattern is seen for etiolated leaves and roots. The plastid fraction contributed over 95 % of the total ferrochelatase activity, but in both cases there was measurable activity in mitochondria that was a greater percentage of the total than the amount of plastid marker in the organelle. The yield of root plastids was much less than from leaves, most probably due to the fact that the starch-containing amyloplasts are more easily ruptured during the initial homogenization.

Table 5 Average ferrochelatase activities per g of fresh weight in plastids and mitochondria from pea tissues

Ferrochelatase activity was measured using the Co-deuteroporphyrin assay, and the total activity contributed by each organelle was calculated using marker enzyme data such as that presented in Table 4. Data are means ± S.E.M. from at least three experiments in root and etiolated tissue, and eight experiments in green tissue. In order to compare between experiments the activities were normalized to fresh weight of starting plant tissue.

Tissue	Ferrochelatase activity (nmol · min^{-1} · g^{-1})	Relative contribution (%)
Green leaves		
Plastids	2.654 ± 1.2	98.7
Mitochondria	0.034 ± 0.02	1.3
Etiolated leaves		
Plastids	0.551 ± 0.34	92.3
Mitochondria	0.046 ± 0.02	7.7
Roots		
Plastids	0.891 ± 0.4	97.2
Mitochondria	0.026 ± 0.01	2.8

We carried out these experiments several times, and obtained very similar results in each case; the averaged results are presented in Table 5, expressed per g of fresh weight, since the amount of starting tissue varied. Several conclusions can be drawn from these data. Firstly, the highest activity was found in chloroplasts, which presumably reflects the requirement for haem synthesis of photosynthetic cytochromes. The activity in etioplasts was about 5-fold lower, correlating with the observed light-induced increase in transcript levels for both *AtFC-I* and *AtFC-II* in *Arabidopsis* [18,19] and *CsFeC2* in cucumber [22], and with the observation that, in tobacco leaves, ferrochelatase activity increases during the light period [40]. Interestingly, the activity in root plastids

Table 4 Relative activity of ferrochelatase in plastids and mitochondria by use of marker enzyme recoveries

Activities of a plastid marker, ALAD [14], and a mitochondrial marker, NAD-ME [31], in total homogenate and organelle fractions of different pea tissues were determined, together with ferrochelatase (FC) activity in the organelles. One experiment for each tissue is illustrated. The level of cross-contamination of plastid and mitochondrial fractions was $\leqslant 3$ %. After determining the recovery of the marker enzymes in the organelles, and assuming the same recovery for ferrochelatase, it was possible to calculate the activity of ferrochelatase in each organelle in the total homogenate.

	Measured activity		Calculated recoveries (%)			Calculated FC in total homogenate (nmol · min^{-1})	
Tissue	ALAD (μmol · h^{-1})	NAD-ME (μmol · min^{-1})	ALAD	NAD-ME	Measured FC activity (nmol · min^{-1})	Plastids	Mitochondria
Green leaves (45 g)							
Total homogenate	23.4	17.8	100	100		185.0	0.543
Plastids	2.29	0.25	9.8	1.4	18.2		
Mitochondria	0.008	5.04	0.03	28.0	0.152		
Etiolated leaves (26 g)							
Total homogenate	3.65	8.3	100	100		19.5	0.858
Plastids	0.19	0.23	5.2	2.7	1.02		
Mitochondria	0.011	1.98	0.3	24.0	0.206		
Roots (20 g)							
Total homogenate	0.997	6.86	100	100		22.4	0.3
Plastids	0.012	0.21	1.2	3	0.226		
Mitochondria	0.005	2.47	0.5	35	0.105		

Table 6 Latency and protease protection of ferrochelatase activity in mitochondria from green leaves of pea

Ferrochelatase (FC), NAD-ME and ALAD activities and chlorophyll content in mitochondria purified from the equivalent of 4 g of green pea leaves. Assays were carried out using lysed mitochondria (standard conditions), and the percentage recoveries in the mitochondria are given in parentheses. In addition, enzymes were assayed in mitochondria without lysis, or after trypsin treament (followed by addition of inhibitor and lysis). n.d., not detectable.

Fraction	FC activity (nmol · min^{-1})	NAD-ME activity (μmol · min^{-1})	ALAD activity (μmol · h^{-1})	Chlorophyll (mg)
Total homogenate	6.3*	1.72	2.88	58.9
Lysed mitochondria (standard assay)	0.38 (6%)	0.71 (41%)	0.01 (0.3%)	0.01 (0.02%)
Unlysed mitochondria	0.09	n.d.	0.01	0.01
Trypsin-treated mitochondria	0.35	0.15	0.005	0.01

* Calculated ferrochelatase activity in chloroplasts + mitochondria in total homogenate.

was greater than in etioplasts, although the significance of this for haem synthesis is not yet clear.

In contrast with the marked differences in ferrochelatase activities in plastids from the three tissues, the activity in mitochondria varied very little, suggesting that the requirement for mitochondrial haem synthesis remained relatively constant. Consequently, the level of ferrochelatase in mitochondria relative to that in plastids varied between 1.3% in green leaf tissue and 7.7% in etiolated leaves, with 2.8% in roots. Nevertheless, these results demonstrate that ferrochelatase in mitochondria is never more than a small fraction of the total activity in the cell. For comparison, we measured protogen oxidase activity in chloroplasts and mitochondria from green pea leaves using the method described in [30], and obtained mean values of 0.022 nmol · min^{-1} · g of fresh weight^{-1} in chloroplasts and 0.011 nmol · min^{-1} · g of fresh weight^{-1} in mitochondria. For this enzyme, therefore, one-third of the cellular activity is present in mitochondria.

The relative proportion of ferrochelatase in mitochondria is of the same order of magnitude as the amount of contaminating plastid marker in the mitochondrial preparations, although in all experiments the calculated proportion of ferrochelatase was greater than the plastid contamination value. Table 6 shows an example, in which levels of contaminating chlorophyll and plastid marker (ALAD) were very low ($< 0.05\%$), whereas up to 6% of the ferrochelatase was recovered in the mitochondria. The most likely form of contamination of the mitochondria is with broken plastids that form bands just above the mitochondria on the Percoll gradients. It is therefore possible to distinguish between the presence of ferrochelatase due to contaminating plastids from that in mitochondria by carrying out a latency experiment, where the mitochondria are assayed intact, omitting the usual hypotonic lysis step to break the mitochondrial membranes and allow access to the substrates. The data in Table 6 show that ALAD activity was the same in lysed and unlysed mitochondria, and was reduced by trypsin treatment. In contrast, NAD-ME was completely latent, indicating that it was contained within a membrane. Similarly, the unlysed sample has less than 25% of the ferrochelatase activity measurable in lysed mitochondria, whereas it was not sensitive to protease treatment, indicating that the enzyme was protected by a membrane. These data are consistent with the presence of ferrochelatase within pea mitochondria, albeit in a much lower proportion than found in plastids.

DISCUSSION

Ferrochelatase is an essential biosynthetic enzyme: the requirement for haem in respiratory and photosynthetic cytochromes is basal to plant metabolism. It is also vital for many other haemoproteins, including catalase, peroxidase and cytochrome P450s, which have major roles in the cell's response to environmental stress. Furthermore, the position of ferrochelatase at the branchpoint of tetrapyrrole biosynthesis makes it important for the regulation of the flux through the pathway to the major end products, chlorophyll, haem and phytochromobilin.

Although in recent years much work has been done on the molecular biology of ferrochelatases in higher plants [18,19,21–23], the biochemical aspects have been less well studied. We wanted to determine the relative roles of plastids and mitochondria in the synthesis of haem within the plant cell. To do this, we developed an assay for the enzyme that is sensitive, reproducible and quantitative, by modifying a method described for measuring ferrochelatase in the red alga *C. caldarium*, which uses the non-endogenous substrates deuteroporphyin IX and Co^{2+}. The specificity of the assay was confirmed by the demonstration that *N*-methylprotoporphyrin, first reported as a potent inhibitor of mammalian ferrochelatase [37], also inhibits the Co-deuteroporphyrin assay of the enzyme in isolated pea organelles, with an IC_{50} value of 1 nM determined for chloroplasts. This is similar to the value of 4 nM determined for the recombinant cucumber ferrochelatase, measured using the pyridine haemochromogen assay [21]. We also found that pea chloroplast ferrochelatase activity was reduced in the presence of ATP. This is interesting given the fact that the other enzyme at the branchpoint in the tetrapyrrole biosynthesis pathway, Mg-chelatase, requires ATP for activity [38]. During the greening of etiolated leaf tissue, there is an increase in the activity of the pathway as a whole to provide intermediates for the massive increase in chlorophyll synthesis. However, there is no concomitant increase in haem production, indicating that Mg-chelatase competes effectively with ferrochelatase for their common substrate, protoporphyrin IX. A model for the role of ATP in this regulation has been proposed [41]. An alternative explanation may come from the observation that one of the three subunits of Mg-chelatase, subunit H, from both *Synechocystis* PCC6803 and *Rhodobacter sphaeroides*, binds deuteroporphyrin [42], and it is a substrate for the enzymes. We have also been able to use deuteroporphyrin to assay Mg-chelatase in pea chloroplasts (J. E. Cornah and A. G. Smith, unpublished work). It is possible therefore that the addition of ATP to the assay simply stimulates the Mg-chelatase reaction, rather than specifically inhibiting ferrochelatase.

The kinetic characteristics of ferrochelatase from pea chloroplasts were comparable with those determined for the enzyme from other higher plants [21,36] and also with the activity found in pea mitochondria. Additionally, the relative specific activities of ferrochelatase (i.e. per mg of protein) in the two organelles

from green leaves (Table 3) were similar to that determined in tobacco leaves [23]; that is, three to five times higher in chloroplasts than mitochondria. Similarly, in roots, there is higher specific activity in plastids than mitochondria, whereas in etiolated leaves the specific activity in the two organelles is essentially the same (Table 3). This indicates that light induces ferrochelatase activity in leaves, which is consistent with the light induction of gene expression of *AtFC-I* and *AtFC-II* genes in *Arabidopsis* [18,19] and ferrochelatase-2 in cucumber [22], and the increase in ferrochelatase activity in tobacco leaves in the light period [40]. However, light has no effect on the level of ferrochelatase activity in mitochondria.

When the ferrochelatase activities are calculated by reference to the recovery of reporter enzymes (Tables 4 and 5), a different picture emerges. This allows a direct comparison of activity in plastids and mitochondria within a unit mass of tissue, effectively within a single cell. In all three tissues ferrochelatase activity in plastids was more than ten times that in mitochondria, suggesting that this organelle has the primary role in synthesizing haem within the cell. In green leaves, this might be expected to be the case since the haem in the photosynthetic cytochromes would constitute the major fraction in the cell. In roots, however, the major pool of haem would be outside the plastid, and yet the plastids had some 15 times more activity than the mitochondria. Our results are highly suggestive that mitochondria play a minor role in overall haem synthesis in the cell, possibly only for the provision of haems for respiratory cytochromes. Haem for other subcellular compartments, such as the endoplasmic reticulum or peroxisomes, is likely to be synthesized within the plastid and then exported. Studies with developing chloroplasts from greening cucumber cotyledons have shown that they are capable of haem efflux [43]. Plant mitochondria are therefore quite different from animal and yeast mitochondria, where all cellular haem is synthesized, including that for export to the rest of the cell.

Indeed, given the very small proportion of total cellular ferrochelatase activity calculated to be in mitochondria, it is conceivable that this is due solely to contamination by plastids. This possibility was raised by Lister et al. [25], when they found that mitochondria from *Arabidopsis* or soya bean were not able to import the AtFC-I precursor protein, although pea mitochondria did, as had been shown previously [24]. Because pea mitochondria (but not those from *Arabidopsis* or soya bean) also imported the small subunit of Rubisco, a bona fide chloroplast protein, it was likely that the former had lost some specificity, so that the import of AtFC-I precursor may be artifactual. However, our data and those of others are incompatible with the absence of ferrochelatase from plant mitochondria. Firstly, although the activity we measured was close to that of contaminating plastid marker enzymes, the latency and protease-protection data (Table 6) indicate that ferrochelatase was enclosed within a membrane, whereas the plastid marker enzyme was not. Furthermore, when specific activity was considered, plastids and mitochondria from etiolated pea leaves had essentially identical levels (Table 3). This could not be explained by contamination of plastids in the mitochondrial fraction. Even in green leaves, the measured specific activity in mitochondria would require contamination by about 20 % of the plastid marker, which is much greater than the 0.1–2 % seen (Tables 4 and 6). Similar relative specific activities have been reported for barley and tobacco [5,23]. Lastly, and most compellingly, there is clearly protogen oxidase activity in plant mitochondria [7–9], and protogen oxidase protein has been detected in tobacco leaf mitochondria by Western-blot analysis [10]. The product of this enzyme, protoporphyrin IX, generates oxygen radicals in the presence of light, resulting in lipid peroxidation. It is the accumulation of protoporphyrin IX in the plasma membrane that is responsible for the photobleaching properties of the diphenyl ether herbicides [8,9,12,17]. It would therefore seem to be essential for ferrochelatase activity to be present in mitochondria to ensure that toxic levels of protoporphyrin IX never accumulate.

As well as providing the means to quantify accurately the relative amounts of ferrochelatase within a single cell, the Co-deuteroporphyrin assay developed here was also used to determine ferrochelatase activity in different tissues from several higher plant sources (Table 2). The specific activity in leaves from all plants tested was similar, but, interestingly, we consistently observed the highest activity in flowers. This correlates with our observations that the promoters of both *AtFC-I* and *AtFC-II* are more active in flowers than in leaves (D. P. Singh, J. E. Cornah and A. G. Smith, unpublished work). This high level of ferrochelatase activity in flowers may be due to high numbers of mitochondria in flowers, particularly anthers [44]. In addition, some phytochromes are highly expressed in flowers, particularly in petals [45], so increased haem production may be required to provide the precursor for phytochromobilin synthesis.

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