A Single Precursor Protein for Ferrochelatase-I from Arabidopsis Is Imported in Vitro into Both Chloroplasts and Mitochondria*

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Ferrochelatase is the last enzyme of heme biosynthesis and in higher plants is found in both chloroplasts and mitochondria. We have isolated cDNAs for two isoforms of ferrochelatase from Arabidopsis thaliana, both of which are imported into isolated chloroplasts. In this paper we show that ferrochelatase-I is also imported into isolated pea mitochondria with approximately the same efficiency as into chloroplasts. Processing of the precursor was observed with both chloroplast stroma and mitochondrial matrix extracts. This was inhibited by EDTA, indicating it was due to the specific processing proteases. The specificity of import was verified by the fact that the mitochondrial preparation did not import the precursor of the light-harvesting chlorophyll a/b protein precursor or the precursor of porphobilinogen deaminase, an earlier enzyme of tetrapyrrole biosynthesis, both of which are exclusively chloroplast-located. Furthermore, import of ferrochelatase-I precursor into mitochondria was inhibited by valinomycin, but this had no effect on its import into chloroplasts. Thus a single precursor molecule is recognized by the import machinery of the two organelles. The implications for the targeting of ferrochelatase in a possible protective role against photooxidative stress are discussed.

Correct targeting of nuclear-encoded proteins within the eukaryotic cell is essential for its function and for the biogenesis of the various organelles. The majority of proteins destined for the mitochondria or chloroplasts are synthesized initially as precursors with N-terminal extensions. These serve as transit peptides to direct the protein to receptors on the surface of the organelle and are generally removed during or after import by specific processing proteases. Analysis of each class of transit peptide has revealed that there is little conservation at the primary sequence level either in composition or in length, although some general features have been identified, such as the fact that mitochondrial presequences frequently form amphiphilic α-helices (1). It is generally considered that they are very specific and that the receptor machinery on the outer membranes of chloroplasts and mitochondria is able to discriminate between bona fide precursors and those which reside in another organelle. For example, the Nicotiana plumbaginifolia transit peptide from the β subunit of F1-ATPase will direct proteins to plant mitochondria in vitro (2, 3) or in vivo (4), but not to chloroplasts. Similarly, chloroplast precursors such as the 33-kDa subunit of the photosynthetic water-splitting complex (OEC33) (3) or the chlorophyll a/b-binding protein (2) are not targeted to plant mitochondria.

Conversely, there is evidence that some “mis-sorting” can occur with chloroplast transit peptides into fungal mitochondria and vice versa. For example, the transit peptide of the small subunit of ribulose-bisphosphate carboxylase (SSU) from Chlamydomonas directed the import of passenger proteins into yeast mitochondria both in vivo and in vitro (5). In a reciprocal experiment, Huang et al. (6) demonstrated that the yeast cytochrome oxidase subunit Va (COXVa) transit peptide directed chloramphenicol acetyltransferase into both chloroplasts and mitochondria in transgenic tobacco. However, both systems use artificial fusion proteins in heterologous systems, and both presequences used were atypical: that of the Chlamydomonas SSU has the potential to form an amphipathic α-helix more typical of mitochondrial transit peptides (7), whereas the yeast COXVa sequence was predicted to form an antiparallel β-sheet (8). Further, since fungi do not possess chloroplasts, there is no need for their mitochondria to have mechanisms to discriminate between precursor proteins, so these data do not necessarily prove mistargeting of proteins between chloroplasts and mitochondria in vivo.

In contrast, Creissen et al. (9) have reported that expression of the cDNA for pea glutathione reductase in transgenic tobacco plants resulted in the targeting of the enzyme both to chloroplasts and to mitochondria. They were also able to show that this targeting was due to the transit peptide, since alone it was able to target a bacterial phosphinothricin acetyltransferase protein to both organelles in vivo. This is not mistargeting, however. Glutathione reductase is found in chloroplasts, mitochondria, and the cytosol (10), but it is encoded by a single nuclear gene in peas, so the translated protein must contain all the information needed for targeting to these three subcellular locations.

Ferrochelatase (EC 4.99.1.1) is the last enzyme of heme biosynthesis, and its activity has been detected in both mitochondria and plastids (11). Using functional complementation of a yeast mutant defective in the enzyme, we isolated a single cDNA clone (AF3) for ferrochelatase from Arabidopsis thaliana (12). The precursor protein encoded by the AF3 cDNA was shown to be targeted to chloroplasts in vitro and processed to the mature size. Furthermore, it was processed by pea stromal extract, but not by maize mitochondrial extract nor by purified mitochondrial processing peptidase from Neurospora. We therefore assumed that this was the chloroplast isoform of the enzyme. Subsequently, we isolated an additional 27 cDNA

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1 The abbreviations used are: SSU, small subunit of ribulose-bisphosphate carboxylase; COX, cytochrome oxidase; PAGE, polyacrylamide gel electrophoresis; NAD-ME, NAD-malic enzyme; LHCP, light-harvesting chlorophyll protein; Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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clones by the same method, which fell into two groups on the basis of restriction maps and Southern hybridization. The larger group, comprising some 22 clones, was essentially the same as the original AF3 clone and subsequently was found to be encoded by the same gene, designated ferrochelatase-I. The other group of 5 cDNAs encoded a ferrochelatase (ferrochelatase-II) with 70% amino acid similarity to the ferrochelatase-I protein. Since we had demonstrated that ferrochelatase-I was targeted to chloroplasts, we expected that ferrochelatase-II was the mitochondrial isoform. However, all attempts to import the precursor protein into isolated mitochondria were unsuccessful. Instead, the protein was imported into chloroplasts and processed to the mature size, with an efficiency similar to that of the ferrochelatase-I precursor. Since we had screened over 10^6 clones in our functional complementation experiment and found a total of 27 independent ferrochelatase cDNAs, it was unlikely that there would be a third isoform of the enzyme. This prompted us to investigate further the targeting of ferrochelatase-I. In this paper we describe experiments which demonstrate that the ferrochelatase-I protein is dual-targeted to both plant mitochondria and chloroplasts in vitro.

EXPERIMENTAL PROCEDURES

Materials—[35S]Methionine (1400 Ci/mmol) was obtained from Amersham International. The riboprobe transcription kit and wheat germ and rabbit reticulocyte lysate translation systems were supplied by Promega. DNA- and RNA-modifying enzymes, SP6 RNA polymerase and T7 RNA polymerase, were obtained from Boehringer Mannheim and used according to the manufacturer's instructions. mGppG was from Pharmacia, thermolysin was from Sigma, and Proteinase K from Promega. DNA- and RNA-modifying enzymes, SP6 RNA polymerase and T7 RNA polymerase, were obtained from Boehringer Mannheim and used according to the manufacturer's instructions. mGppG was from Pharmacia, thermolysin was from Sigma, and Proteinase K from Promega. DNA- and RNA-modifying enzymes, SP6 RNA polymerase and T7 RNA polymerase, were obtained from Boehringer Mannheim and used according to the manufacturer's instructions. mGppG was from Pharmacia, thermolysin was from Sigma, and Proteinase K from Promega.

Plant Growth—Peas (Pisum sativum L. var Feltham First) for preparation of chloroplasts and mitochondria, were sown in Levington potting compost and grown under a cycle of 16-h light, 8-h dark at an ambient temperature of 20–25 °C for 7–8 days (chloroplasts) or 13–15 days (mitochondria) before harvesting.

Production of Precursor Proteins in Vitro—Plasmids pGE3, pA222, pLHCP II, and pMAL were used to produce radiolabeled precursor proteins in vitro. The plasmids, purified on cesium chloride gradients of a wide range of other plant precursor proteins (25). Mitochondria were prepared from 12–14-day-old pea leaves according to Fang et al. (20). For mitochondrial import, each assay contained purified mitochondria (equivalent to 50 μg of mitochondrial protein), 0.3 mM mannitol, 5 mM t-methionine, 80 mM KCl, 2 mM MgCl2, 4 mM sodium phosphate, 20 mM Tris-NaOH, pH 7.2, 2 mM dithiothreitol, 2 mM ADP, 4% (v/v) fatty acid-free bovine serum albumin, and 8–10 μl of reticulocyte lysate translation product in a total volume of 200 μl. As for chloroplast import, three reaction tubes were set up: one which was treated with protease (30 min on ice with 10 μg/ml Proteinase K, terminated with 3 mM phenylmethylsulfonyl fluoride), one which was treated with protease (30 min on ice with 0.5% (v/v) Triton X-100, and one untreated. Intact mitochondria were isolated by centrifugation through a 20% sucrose cushion (20% (v/v) sucrose, 20 mM Tris-NaOH, pH 7.2) at 2000 × g for 1–2 min.

For the mitochondrial processing assay, mitochondria were prepared as above except that EDTA was excluded from all the isolation buffers. Up to 500 μg of mitochondrial protein was incubated in mitochondrial processing buffer (25 mM Tris-HCl, pH 8.0, 1% (v/v) Triton X-100, 100 mM NaCl, 0.1 mM MnCl2, and 0.1 mM MgCl2) for 10 min on ice. For the EDTA control, 0.1 mM EDTA was included in the reaction. After flash-freezing in liquid nitrogen followed by thawing on ice to lyse the mitochondria, 3 μl of translation mixture and 1 μl of phenylmethylsulfonyl fluoride were added, and the processing reaction was incubated at 30 °C for 30 min. Samples were analyzed by SDS-PAGE and fluorography as for chloroplast assays.

Enzyme Assays—Samples taken at various stages during the purification of mitochondria from pea leaves were assayed for the activity of NAD-ME, which is present in the mitochondrial matrix (21), by the method of ap Rees et al. (22), and adenylate kinase, which is present in the inner membrane space (23), as described by Smith et al. (24).

RESULTS

Import of Precursors into Isolated Pea Mitochondria—The small size of Arabidopsis plants means that they are unsuitable as a source of chloroplasts and mitochondria for import experiments, since it is essential to prepare them from young actively growing tissue in high yield. Instead we used peas, not least because they have been used extensively to study import of a wide range of other plant precursor proteins (25). Mitochondria were isolated from 15-day-old pea leaves and purified on two successive Percoll gradients, as described under "Experimental Procedures." They were essentially free of chlorophyll and sustained high rates of oxygen uptake (data not shown). The mitochondria were then tested for import competence using the precursor for NAD-malic enzyme (NAD-ME) from maize, which is a bona fide mitochondrial protein and which had been demonstrated to be imported into isolated maize mitochondria (16). Translation and transcription of the pMAL plasmid encoding NAD-ME produced a major polypeptide band of 62 kDa (Fig. 1A, lane 1), the predicted molecular size of the NAD-ME precursor. Incubation of this precursor with the mitochondria resulted in a 59-kDa mature protein (lane 2). After import of the mitochondria, Proteinase K to digest any protein on the outside of the organelle, the NAD-ME mature protein remained (lane 3), but it was completely digested by Proteinase K in the presence of Triton X-100 (lane 4), indicating that it was not protease-resistant in the absence of a membrane. This result demonstrates that the NAD-ME precursor was successfully imported into the isolated pea mitochondria.
Specific stromal processing peptidase, which requires metal and ferrochelatase-I were processed to the mature size (lane 2) prior to disruption of the membrane by Triton X-100 (lane 3). The precursor protein disappeared after treatment of the mitochondrial extract with the same three precursors. Interestingly, it proved difficult to demonstrate processing in vitro of the precursor for maize NAD-ME using the published conditions (28), which we had demonstrated previously were suitable for processing of yeast COXIV (12). However, by increasing the amount of mitochondrial protein from 100 to 500 μg, it was possible to observe processing of pre-NAD-ME in vitro, albeit very inefficiently. The size difference between the precursor and mature forms of NAD-ME is small (cf. Fig. 1A), but the sample with mitochondrial extract (Fig. 2B, lane 3) contains a second faint protein (arrowed) underneath the 62-kDa precursor. The second band is not produced if EDTA is included in the incubation (lanes 4–6), indicating that it was a substrate for stromal processing peptidase. A similar processing was inhibited by the presence of EDTA (lane 5) containing standard import reaction. However, in the experiments described above, ferrochelatase-I precursor is clearly processed during import into pea mitochondria. We therefore carried out further experiments to investigate the susceptibility of the precursor to organelle processing peptidases: Firstly, concentrated stromal extract from pea chloroplasts (equivalent to 15 μg of chlorophyll) was incubated with the precursors for LHCP, NAD-ME, and ferrochelatase-I (Fig. 2A). Both LHCP and ferrochelatase-I were processed to the mature size (lanes 2 and 5), and this processing is inhibited by the addition of 0.1 mM EDTA (lanes 3 and 6), indicating that the cleavage is by the specific stromal processing peptidase, which requires metal ions for activity (26). In contrast, the precursor for NAD-ME appeared unaffected by chloroplast stromal extract, in the presence or absence of EDTA (lanes 8 and 9), suggesting that it was not a substrate for stromal processing peptidase. A similar result was obtained using the precursor for the adenine-nucleotide transporter from maize (27) (data not shown).

We then conducted the equivalent experiment using pea mitochondrial extract with the same three precursors. Surprisingly, it proved difficult to demonstrate processing in vitro of the precursor for maize NAD-ME using the published conditions (28), which we had demonstrated previously were suitable for processing of yeast COXIV (12). However, by increasing the amount of mitochondrial protein from 100 to 500 μg, it was possible to observe processing of pre-NAD-ME in vitro, albeit very inefficiently. The size difference between the precursor and mature forms of NAD-ME is small (cf. Fig. 1A), but the sample with mitochondrial extract (Fig. 2B, lane 3) contains a second faint protein (arrowed) underneath the 62-kDa precursor. The second band is not produced if EDTA is included in the incubation (lanes 4–6), indicating that it was a substrate for stromal processing peptidase. A similar processing was inhibited by the presence of EDTA (lane 5) containing standard import reaction. However, in the experiments described above, ferrochelatase-I precursor is clearly processed during import into pea mitochondria. We therefore carried out further experiments to investigate the susceptibility of the precursor to organelle processing peptidases: Firstly, concentrated stromal extract from pea chloroplasts (equivalent to 15 μg of chlorophyll) was incubated with the precursors for LHCP, NAD-ME, and ferrochelatase-I (Fig. 2A). Both LHCP and ferrochelatase-I were processed to the mature size (lanes 2 and 5), and this processing is inhibited by the addition of 0.1 mM EDTA (lanes 3 and 6), indicating that the cleavage is by the specific stromal processing peptidase, which requires metal ions for activity (26). In contrast, the precursor for NAD-ME appeared unaffected by chloroplast stromal extract, in the presence or absence of EDTA (lanes 8 and 9), suggesting that it was not a substrate for stromal processing peptidase. A similar result was obtained using the precursor for the adenine-nucleotide transporter from maize (27) (data not shown).

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the ferrochelatase-I precursor is found associated with the mitochondria, there is no processing to the mature size (Fig. 3A, lane 5), and the precursor is completely digested by treatment with protease (data not shown). In contrast, valinomycin had no effect on the efficiency of import of ferrochelatase-I into isolated pea chloroplasts (Fig. 3B, compare lanes 2 and 4). Fig. 3C shows the results of import of ferrochelatase-I into both chloroplasts and mitochondria analyzed on the same gel to compare the size of the processed proteins. In each case a 42-kDa band is found, which corresponds to the expected size of the mature protein (12). The additional band of 38 kDa, which is observed after protease treatment of mitochondria, is not found after import into chloroplasts. This was investigated further as described below.

From the experiments so far, there was little apparent difference between the capacity of mitochondria and chloroplasts to import the ferrochelatase-I precursor. To investigate this further, the time course of import into the two organelles was studied. Import assays were carried out essentially as before, with a total volume of 600 μl. Aliquots of 100 μl were removed at set time intervals and treated with protease, and the organelles were reisolated. The products were analyzed by SDS-PAGE and fluorography, and the results were quantified by densitometry. Typical data are shown in Fig. 4. For chloroplasts, there was a steady increase in protein accumulated for the first 10 min, but then it plateaued. After 30 min there was a decline, suggesting that there was degradation of the imported protein. Similarly, there was a linear increase of protein into mitochondria up to 20 min, but a reduction was seen after 30 min. In this particular experiment more of the mature ferrochelatase-I (as a percentage of precursor added) was imported into mitochondria than into chloroplasts, but this varied from experiment to experiment, depending on the particular organelle preparation and translation reaction. For both chloroplasts and mitochondria, the amount of protein imported varied from about 5% to 35% of precursor added. Thus the efficiency of import would appear to be the same into the two organelles. This is clearly indicated in the direct comparison shown in Fig. 3C.

Origin of the Additional 38-kDa Band after Protease Treatment of Mitochondria—From Fig. 3C, it can be seen that the additional band of 38 kDa found after protease treatment of mitochondria is not produced after import into chloroplasts, either before or after protease treatment. Neither was it observed after processing with mitochondrial extract (Fig. 2B). This suggests strongly that it is generated as a result of treatment of mitochondria with Proteinase K, rather than a product of the mitochondrial import. Further evidence for this explanation is that the amount of the 38-kDa band is greater after a 20-min incubation in Proteinase K than after 10 min (Fig. 3A, compare lanes 3 and 4). It was also seen when thermolysin was used as the protease (data not shown). This implies that the ferrochelatase-I protein imported into the pea mitochondria may have a small region which is accessible outside the organelle membrane. To investigate this further, we determined the extent to which the mitochondria we isolated had lost their outer membrane, resulting in mitoplasts, by following the distribution of two mitochondrial enzymes during the fractionation procedure. NAD-malic enzyme is compartmentalized in the mitochondrial matrix (21) whereas adenylate kinase is located in the intermembrane space (23). The activity of both these enzymes was determined in various fractions generated during the preparation of mitochondria for import, and Table I
presents these results expressed as a percentage of the total activity present in the total homogenate. The activity of adenylate kinase recovered in the mitochondrial pellet was only 8.5% of the activity in the total homogenate, compared with 48% for NAD-malic enzyme. Both enzyme activities were reduced further in the Proteinase K-treated mitochondria, but adenylate kinase was more sensitive, indicating that a large proportion of the mitochondria were mitoplasts, and so any protein present in the intermembrane space would be accessible to exogenously added protease. It is likely therefore that the additional 38-kDa band is the result of protease cleavage of a region of ferrochelatase-I protein which is orientated into this part of the mitochondrion.

Verification of the Specificity of Mitochondrial Import—Despite the considerable evidence which we had obtained to demonstrate that ferrochelatase-I is imported into plant mitochondria, there is still the possibility that this is an artifact of the in vitro system. We therefore used a precursor for another tetrapyrrole biosynthesis enzyme, porphobilinogen deaminase, which had previously been shown to be imported into pea chloroplasts (14) and which had been determined to be located exclusively within the plastids (30). Fig. 5 shows the results of incubation of the precursor of Arabidopsis porphobilinogen deaminase with isolated pea mitochondria. The precursor (45 kDa) is found associated with the mitochondria, together with another slightly smaller protein of 43 kDa (lane 3). This is not the mature protein, which is 40 kDa (14). Neither of the bands in the total import reaction is protease-resistant (lane 2), indicating that they are on the outside of the mitochondrial membrane, completely accessible to the protease. Furthermore, incubation of the precursor protein with mitochondrial extract (equivalent to 500 μg of protein) has no effect (lane 4). The chloroplast-located porphobilinogen deaminase is therefore not imported or processed by our isolated pea mitochondria. These data lend further support to the conclusion that the targeting of ferrochelatase-I to pea mitochondria is genuine.

**Table I**

| Fraction                        | NAD-malic enzyme | Adenylate kinase |
|--------------------------------|------------------|------------------|
| Total homogenate                | 100.0            | 100.0            |
| 3300 × g supernatant            | 100.0            | 63.5             |
| 3300 × g pellet                 | 0.0              | 0.0              |
| 10,000 × g supernatant          | 0.0              | 0.0              |
| 10,000 × g pellet               | 99.5             | 30.8             |
| Percoll-purified mitochondria   | 48.0             | 8.5              |
| Proteinase K-treated mitochondria| 24.0             | 1.3              |

**Fig. 5. Incubation of the precursor to porphobilinogen deaminase with mitochondria.** Lane 1, translation products (equivalent to one-tenth used in each import reaction) from pAC223 (14); lane 2, incubation with mitochondria followed by treatment with Proteinase K; lane 3, incubation with mitochondria (total import reaction); lane 4, incubation with mitochondria pretreated with valinomycin; lane 5, incubation of translation products with mitochondrial matrix extract (equivalent to 500 μg of protein). The porphobilinogen precursor is 45 kDa, whereas the mature protein is 40 kDa (14). The extra band of 43 kDa seen in lane 3 is therefore not the mature protein and may arise from the action of nonspecific proteases in the mitochondrial preparation.
similar in mitochondria and etioplasts from dark grown barley leaves, and indeed the two isoforms had very similar properties. Direct comparison of activity and import efficiency for ferrochelatase is, however, complicated by the fact that in chloroplasts there is a separate isozyme, ferrochelatase-II (13).

Nevertheless, even without direct evidence of dual targeting of ferrochelatase-I in vivo, the behavior of precursor proteins in import experiments in vitro has generally been assumed to reflect the situation in vivo, and there is no reason why this should not be the case for ferrochelatase-I. Furthermore, although until recently it was assumed that multiple isoforms of an enzyme located in different compartments were encoded by separate nuclear genes, as is the case for superoxide dismutase (33) and malate dehydrogenase (34), examples of proteins targeted to more than one location are becoming more common. For example, the mitochondrial and cytosolic isoforms of rat fumarase (35) and isopentenyl-pyrophosphate:ribose isopentenyltransferase and ATP(CTP):ribose nucleotidyltransferase from yeast (36) are each the products of a single gene, as are the cytosolic and mitochondrial forms of alanine aminocayl-ribose synthetase in Arabidopsis (37). Analysis of transcription start sites in the latter gene showed the presence of two potential AUG codons in some transcripts, suggesting the possibility that the same message could give rise to two different polypeptides. A similar suggestion has been made for pea glutathione reductase (9).

For ferrochelatase-I, alternative transcription or translation cannot be the explanation for the dual targeting we have observed. In general, there is a single radiolabeled protein of 50 kDa in the translation products of pAF3, corresponding to the predicted size of the full-length precursor. Although there is a second AUG in the message, the protein translated from this AUG codon would be 45 amino acids shorter, equivalent to a loss of one AUG. Despite missing the majority of the mitochondrial targeting information, this clone nonetheless was able to rescue a yeast hem15 mutant devoid of ferrochelatase activity. A similar result was obtained with a construct encoding a truncated form of yeast ferrochelatase (38). The authors found that in hem15 cells containing this clone, although there was detectable ferrochelatase activity, this was improperly localized throughout all the membranes of the cell, rather than confined to the matrix side of the inner mitochondrial membrane, which is the normal location of ferrochelatase (39). The amount of heme synthesized in these cells was reduced compared with wild-type, but this was sufficient to allow functional complementation of the mutant, probably because in wild-type yeast, ferrochelatase activity is not limiting for normal heme synthesis (40). Although the Arabidopsis F5 clone may simply represent a truncated cDNA formed during library construction, an intriguing possibility is that this encodes a cytosolic form of ferrochelatase. Recently, ferrochelatase activity has been found associated with the plasma membrane, which also contains measurable activity of the previous enzyme of the tetrapyrrole synthesis pathway, protoporphyirinogen oxidase (41). The product of this enzyme, protoporphyrin IX, is extremely photoreactive, as evidenced by the effectiveness of the diphenylether herbicides which cause this compound to accumulate (42, 43). In treated plants in the light, reactive oxygen species are formed which then cause lipid peroxidation and rapid cell death (44). The presence of ferrochelatase in several cell membranes may thus be a means to ensure that any protoporphyrin IX made under normal circumstances is rapidly metabolized and not allowed to accumulate. In this respect, ferrochelatase would have a similar role to glutathione reductase, which is also an important part of the plant cell's apparatus to deal with oxidative stress.

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