Dual Intracellular Localization and Targeting of Aminoimidazole Ribonucleotide Synthetase in Cowpea[1]

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De novo purine biosynthesis is localized to both mitochondria and plastids isolated from *Bradyrhizobium* sp.-infected cells of cowpea (*Vigna unguiculata* L. Walp) nodules, but several of the pathway enzymes, including aminoimidazole ribonucleotide synthetase (AIRS [EC 6.3.3.1], encoded by *Vupur5*), are encoded by single genes. Immunolocalization confirmed the presence of AIRS protein in both organelles. Enzymatically active AIRS was purified separately from nodule mitochondria and plastids. N-terminal sequencing showed that these two isoforms matched the *Vupur5* cDNA sequence but were processed at different sites following import; the mitochondrial isoform was five amino acids longer than the plastid isoform. Electrospray tandem mass spectrometry of a trypsin digest of mitochondrial AIRS identified two internal peptides identical with the amino acid sequence deduced from *Vupur5* cDNA. Western blots of proteins from mitochondria and plastids isolated from root tips showed a single AIRS protein present at low levels in both organelles. ^{35}S-AIRS protein translated from a *Vupur5* cDNA was imported into isolated pea (*Pisum sativum*) leaf chloroplasts in vitro by an ATP-dependent process but not into import-competent mitochondria from several plant and non-plant sources. Components of the mature protein are likely to be important for import because the N-terminal targeting sequence was unable to target green fluorescent protein to either chloroplasts or mitochondria in Arabidopsis leaves. The data confirm localization of the protein translated from the AIRS gene in cowpea to both plastids and mitochondria and that it is cotargeted to both organelles, but the mechanism underlying import into mitochondria has features that are yet to be identified.

In root nodules of legumes of the tribe Phaseoleae, symbiotically fixed N_2 is assimilated by de novo purine biosynthesis, after which the purines are oxidized to allantoin and allantoic acid (Atkins and Smith, 2000). It is in this form that fixed N is exported in xylem from the nodule to the shoot. The 10 enzymes of the purine biosynthetic pathway have been well studied in relation to their role in cancer metabolism, and the genes encoding them cloned from a wide variety of organisms. However, in plants, far less is known about the enzymes of the pathway, although genes encoding a number have now been isolated (Atkins and Smith, 2000).

The cDNA sequences of all the plant purine biosynthetic (*pur*) genes studied have 5′ extensions relative to the coding regions of the corresponding *Escherichia coli* genes (Senecoff and Meagher, 1993; Chapman et al., 1994; Schnorr et al., 1994, 1996; Kim et al., 1995; Senecoff et al., 1996; Smith et al., 1998). These extensions potentially encode N-terminal, organelle-targeting sequences and indicate that in contrast to other eukaryotes (Gooljarsingh et al., 2001), the pathway is likely to be organelle-localized in plants. This was confirmed in a recent study that showed that in cowpea (*Vigna unguiculata* L. Walp) nodules, both the mitochondria and plastids must have all 10 enzymes of the pathway because each organelle is able to synthesize purines de novo (Atkins et al., 1997). Whether dual localization of the pathway occurs in other plant tissues is not known.

Where enzyme activity is localized to more than one subcellular compartment, there are generally two or more genes encoding different cytosolic precursors, each with targeting information specific for one organelle (for examples, see Rolland et al., 1993; Danpure, 1995; Boston et al., 1996; Lermontova et al., 1997). In contrast, analysis of cDNA clones and Southern blots suggests that in cowpea, some of the purine biosynthetic enzymes are each encoded by a single gene despite their localization to both mitochondria and plastids (e.g. Smith et al., 1998). One such example is aminoimidazole ribonucleotide synthetase (AIRS; EC 6.3.3.1), which catalyzes the ATP-dependent formation of aminoimidazole ribonucleotide from formylglycinamidine ribonucleotide in the fifth step of de novo purine biosynthesis. Arabidopsis AIRS is also the product of a single gene (Senecoff and Meagher, 1993).

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If a dual-localized protein is encoded by only one gene, specific targeting information for a particular organelle can be provided by a number of mechanisms, for example differential transcription or translation and alternative RNA splicing (for extensive review, see Danpure, 1995; Small et al., 1998). An increasing number of proteins have been described in which the same targeting information cotargets the protein to both mitochondria and chloroplasts (Peeters and Small, 2001). These include glutathione reductase from pea (*Pisum sativum*; Creissen et al., 1995), ferrochelatase-I (Chow et al., 1997) from Arabidopsis and a number of amino acyl-tRNA synthetases (for summary, see Peeters and Small, 2001).

The putative N-terminal targeting information for cowpea AIRS possesses characteristics of both mitochondrial presequences and plastid transit peptides, but is not "typical" of either, and there is little evidence for differential transcription or translation of the gene (Smith et al., 1998). In this study, the subcellular localization of AIRS in plastids and mitochondria was confirmed using immunolocalization, and immunoaffinity chromatography was used to purify AIRS separately from isolated cowpea nodule mitochondria and plastids so that their physical characteristics could be compared. In vitro and in vivo targeting experiments with isolated chloroplasts and mitochondria were used to investigate the targeting properties of the cowpea AIRS precursor. In addition, the localization of AIRS in non-nodule tissues from cowpea was investigated.

RESULTS

Localization

Nodule material used for immunolocalization comprised both central infected tissue and cortex (Fig. 1A). Silver-enhanced immunogold-labeling showed that AIRS antisera bound most strongly to the periphery of infected cells in the central zone (Fig. 1B). The rest of the infected cells stained less strongly, and there was negligible staining of uninfected cells or of the cortex. Preimmune serum did not bind significantly to any cells in the sections (Fig. 1C). Electron microscopy confirmed that the peripheral region of infected cells was enriched in plant organelles (Fig. 2). Both mitochondria (26.6 ± 3.2 Au particles field^{-1}) and plastids (44.6 ± 9.2 Au particles field^{-1}) bound AIRS antibodies at a higher frequency than the cytosol (17.9 ± 2.5 Au particles field^{-1}) or bacteroids (11.1 ± 3.0 Au particles field^{-1}; Fig. 2A; frequencies are means ± SE, n = 14). The lower frequency of Au labeling seen with preimmune serum (Fig. 2B) was not specifically associated with any organelle (3 ± 1 Au particles field^{-1} in cytosol, 5.7 ± 1.7 in bacteroids, 3.5 ± 1.3 in plastids, and 5.7 ± 1.8 in mitochondria; data are means ± SE, n = 7).

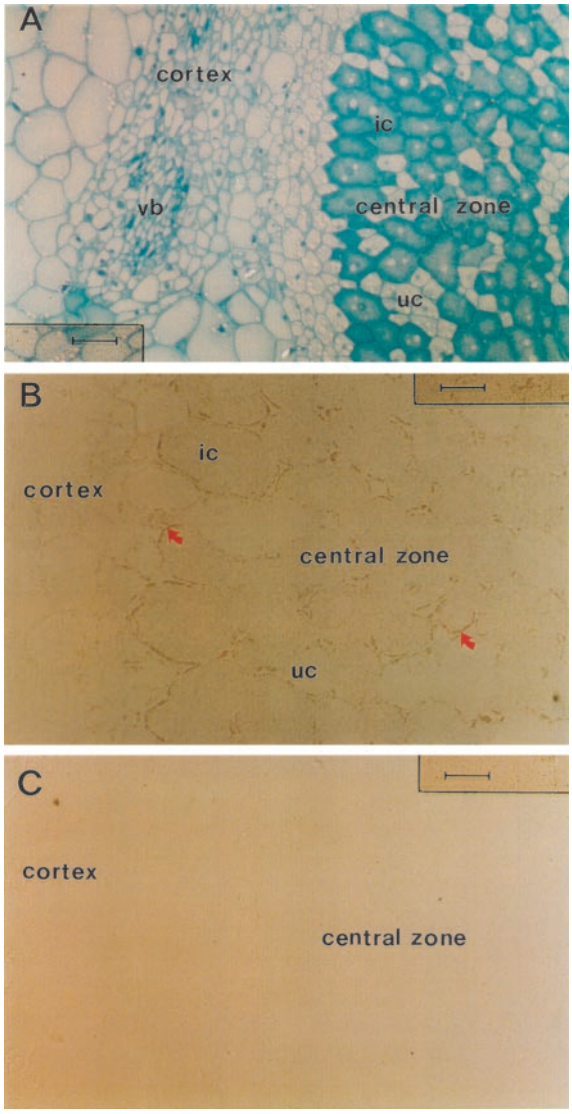

Figure 1. Localization of AIRS in light micrographs of sections of cowpea nodules after Au-immunolabeling with silver enhancement. A, Section stained with methylene blue. B, Unstained section treated with primary AIRS antiserum (ic, infected cell; vb, vascular bundle; uc, uninfected cell). Clusters of silver grains are visible as dark deposits at the periphery of the infected cells in the central zone and especially concentrated around intercellular spaces (red arrows). A much weaker signal is also present across the whole of the infected cells. Staining in the cortex is less dense and where present appears also to be localized to the cell periphery. C, Unstained section treated with preimmune serum at the same dilution (1:1,000, v/v) as the primary AIRS antiserum. There is no visible deposition of silver grains. Bars = 100 μm in A, 40 μm in B and C.

Purification and Partial Characterization of Mitochondrial and Plastid Isoforms

Enzymatically active AIRS protein was isolated separately from both plastids and mitochondria. Intact mitochondria were prepared such that there was no contamination by other plant organelles, bacteroids, or soluble proteins. Plastids and bacteroids cofractionated after Percoll density gradient centrif-

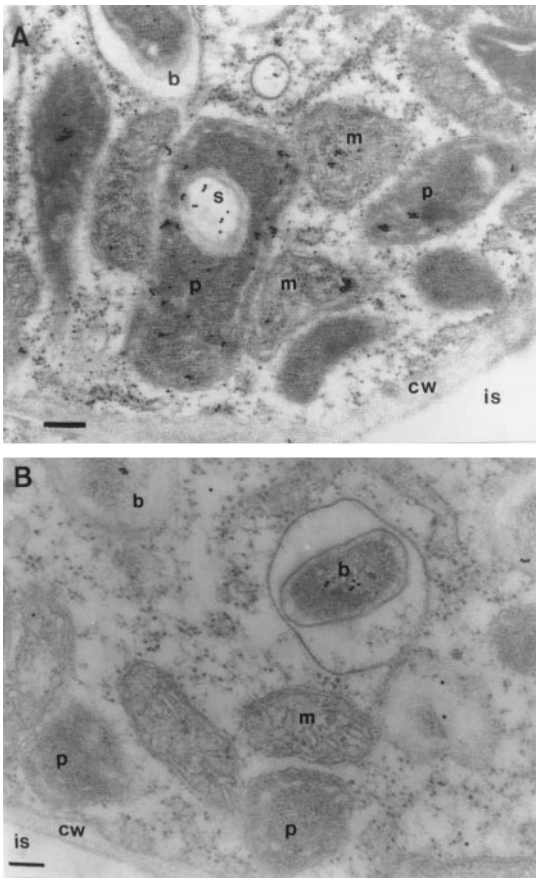

Figure 2. Localization of AIRS in electron micrographs of sections of Au-immunolabeled tissue of cowpea nodules. A, Section treated with primary AIRS antiserum. Note Au particles over the plastids and mitochondria. B, Section treated with nonimmune sera. Note background signal over the bacteroid and absence of signal in mitochondria and plastids. p, Plastid; m, mitochondria; cw, cell wall; b, bacteroid; is, intercellular space; s, starch grain. Bars = 200 nm.

ugation and intact plastids could not be recovered. However, if the bacteroid/plastid fraction was snap frozen in liquid N_2 and thawed, only the plastids were lysed so that their contents could be collected as a soluble extract. Assays of this soluble extract for a bacteroid-marker enzyme, β-hydroxybutyrate dehydrogenase (β-HBD; Shelp et al., 1983), yielded a low level of activity (9 nmol min^{-1} mg^{-1} protein), indicating some small degree of lysis of bacteroids after this freeze-thaw treatment. Lysing the bacteroid pellet by Triton X-100 treatment after removing the soluble plastid extract released the β-HBD activity (91 nmol min^{-1} mg^{-1} protein). Thus at most, the plastid extract might have been contaminated by 10% of the soluble protein from bacteroids. No β-HBD activity could be recovered in the washed mitochondrial suspension whether or not detergent was added. Furthermore, the soluble plastid extract was essentially free of mitochondrial contamination as indicated by a very low level of Glu dehydrogenase activity (marker for plant mitochondria; Shelp et al., 1983). The plastid fraction typically showed 1 to 4 nmol Glu-dependent NAD-reduction min^{-1} mg^{-1} protein compared with 130 nmol Glu-dependent NAD-reduction min^{-1} mg^{-1} protein for the mitochondria fraction. On this basis, the plastid extract might have been contaminated by at most 3% of mitochondrial proteins.

AIRS eluted from the immunoaffinity matrix retained enzymatic activity. Comparing the activity of isolated mitochondria and plastid extracts to that of purified AIRS from each organelle, mitochondrial AIRS was purified 18-fold (yield, 22%) and plastid AIRS 27-fold (yield, 57%). Calculated molecular masses of mitochondrial and plastid AIRS by SDS-PAGE were 36.9 and 36.4 kD, respectively, a difference of 0.5 kD (Fig. 3).

The small mass difference between isoforms was used to test for the presence of each during nodule development. After separation by SDS-PAGE and blotting to nitrocellulose, protein extracts of nodules from 14-, 21-, 28-, and 35-d-old plants were screened with AIRS antisera (Fig. 4). A closely spaced doublet corresponding to mitochondrial and plastid AIRS was present in nodules at each age, indicating that the protein is already present in both organelles at the onset of N_2 fixation (approximately 14 d after germination).

Native PAGE showed that the intrinsic molecular masses of mitochondrial and plastid AIRS were 75.9 and 74.4 kD, respectively, so that in its functional form, the enzyme in each organelle is likely to be a homodimer.

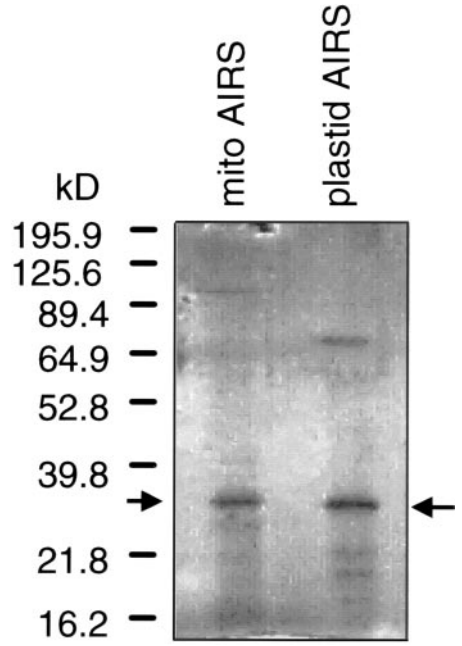

Figure 3. Purified cowpea nodule mitochondrial and plastid AIRS. AIRS that was immunoaffinity purified from mitochondria (*mito AIRS*) and plastids (*plastid AIRS*) was run on an SDS-12% (w/v) polyacrylamide gel and silver stained. Molecular mass markers (kilodaltons) are shown in the left lane. The arrows indicate the two AIRS isoforms that were excised from a blot of the gel for N-terminal sequencing.

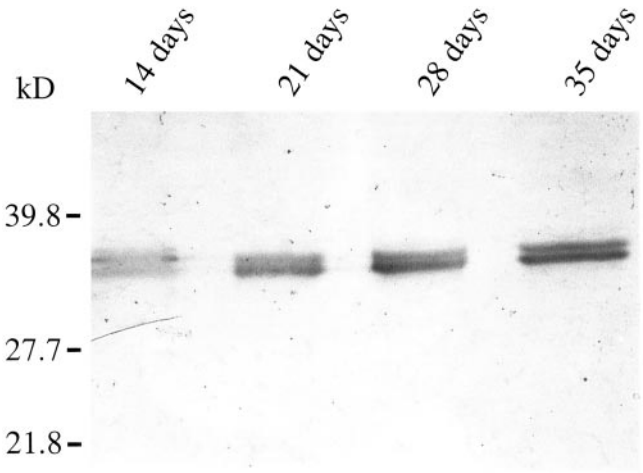

Figure 4. SDS-PAGE analysis of AIRS during nodule development. Extracts of nodules from plants aged 14, 21, 28, and 35 d were separated on a SDS-12% (w/v) polyacrylamide gel and immunostained with anti-AIRS antiserum. Doublets representing mitochondrial and plastid AIRS were present in each lane of nodule extract. The position of molecular mass markers (kilodaltons) is shown to the left of the figure.

The N-terminal sequence for mitochondrial AIRS determined by automated Edman degradation was identical to that deduced from the *Vupur5* cDNA encoding AIRS (Fig. 5A). The plastid sequence was also exactly the same except that residue 6 was not clear, with L and G giving signals of similar strength. The deduced sequence from the cDNA has a G at this position. On the basis of the cDNA sequence, the mature mitochondrial protein (presequence length 61 residues) is five residues longer than the mature plastid protein (transit peptide length 66 residues; Fig. 5B). The calculated difference in molecular mass between mitochondrial and plastid AIRS subunits based on the sequence data is 456 D.

Separation of the proteins extracted from mitochondria isolated and purified from mature cowpea nodules by two-dimensional gel electrophoresis indicated one major and two minor bands following western blotting with AIRS immune serum. After trypsin digestion of the major protein spot, the peptides were analyzed by direct injection into an electrospray ionization-tandem mass spectrometer (MS/MS), and collision induced dissociation of selected peptides. Two peptides were found to have amino acid sequences that matched that deduced from the *Vupur5* cDNA. The larger of these had a M_r = 1,624.78, and the sequence G[I/L]AH[I/L]TGGGFTDN[I/L]PR corresponded to amino acids 291 to 306 in the sequence deduced from *Vupur5*; whereas the smaller, with a M_r = 1,399.58 and a sequence of DAGVD[I/L]DAGAE[I/L]VR, corresponded to amino acids 70 to 83.

A protein band that corresponded in size to nodule AIRS proteins was also identified in western blots of both mitochondria and plastids isolated from the tips of primary seedling roots of cowpea that were probed with AIRS immune serum (Fig. 6). Although the overall amount of AIRS from roots was very small compared with nodules, unlike nodules, in which approximately equal amounts of AIRS protein were recovered from the two organelles, most of the protein in roots was in plastids. Thus the very low level of AIRS recovered in mitochondria could be a result of contamination of the mitochondria preparation with plastids. However, this is unlikely because the media used to wash the mitochondria after isolation ruptures plastids while leaving mitochondria intact.

In Vitro Import of Radiolabeled Proteins

A ^{35}S-labeled protein of approximately 47 kD was produced after translation of the *Vupur5* cDNA (Smith et al., 1998) in a coupled rabbit reticulocyte lysate system (Figs. 7 and 8).

Compounds that block import into organelles, apyrase for chloroplasts and valinomycin for mitochondria, were included as controls in each experiment to determine the degree to which import was achieved through already documented pathways. Apyrase deprives the chloroplast import machinery of ATP, and valinomycin disrupts the electrochemical potential across the inner membrane of mitochondria. In both cases, organelle preparations were treated with proteolytic enzymes after incubation to remove any labeled protein that had not been imported.

Cowpea AIRS and ribulose bisphosphate carboxylase/oxygenase activase (RCA) were imported in an ATP-dependent manner into a protease-protected location in pea chloroplasts and processed to a smaller size, whereas alternative oxidase (AOX) was neither imported nor processed (Fig. 7). The truncated cowpea AIRS precursor that lacked the putative targeting presequence was not imported into pea chloroplasts.

Only AOX was imported and processed in a membrane potential-dependent manner by mitochondria from soybean (*Glycine max*) cotyledons (Fig. 8). The cowpea AIRS protein was also tested for import into mitochondria from potato (*Solanum tuberosum*) tuber,

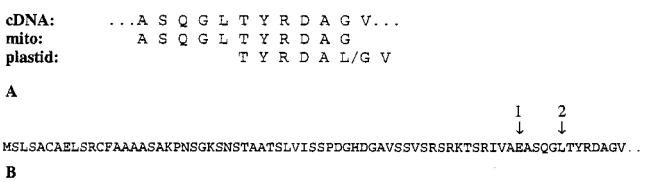

Figure 5. N-terminal sequences of mitochondrial and plastid AIRS. A, Direct N-terminal sequences of purified mitochondrial and plastid AIRS compared with each other and with the cDNA-derived sequence. B, Deduced cleavage sites of the AIRS precursor (cDNA-derived) after import into mitochondria and plastids based on the N-terminal sequences from A. Arrow 1, The mitochondrial cleavage site between E61 and A62; arrow 2, the plastid cleavage site between L66 and T67.

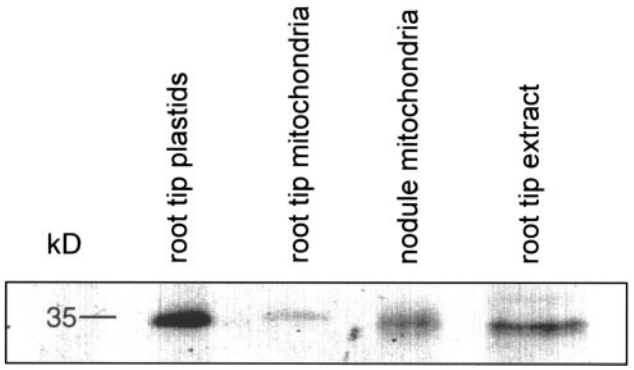

Figure 6. Western blot of AIRS protein separated by SDS-PAGE of extracts of the 3- to 5-mm tip of the primary root of 4-d-old germinated cowpea seedlings or from plastids and mitochondria isolated from this tissue. The polyclonal antisera were generated from recombinant *Vupur5* protein. Nodule mitochondria were prepared as described in "Materials and Methods." Equal amounts of protein were loaded to each lane on the gel. The size marker was calculated from the migration of a series of marker proteins (as in Fig. 3).

etiolated rice (*Oryza sativa*) seedlings, yeast, rat liver, soybean cotyledons, and soybean nodules, but in no case was there evidence for uptake into a protease-protected location (data not shown). AOX was correctly imported and processed by mitochondria from all sources, whereas RCA was not.

Import of AIRS and AOX was also investigated using mitochondria isolated from either cowpea cotyledons or nodules to determine whether a homologous source of mitochondria is required. Mitochondria from cowpea cotyledons gave the same results as those from soybean (data not shown). Mitochondria were isolated from cowpea nodules a number of times, and although assays for O_2 uptake showed that they were intact and that electron transport was coupled with ATP production, they were never import competent. Neither AIRS nor AOX was imported into mitochondria isolated from younger (from 14-d-old plants; data not shown) or more mature nodules (from 35-d-old plants; data not shown).

In Vivo Import Assays

When transiently expressed in Arabidopsis leaves as a fusion with green fluorescent protein (GFP), the first 71 amino acids of the AIRS protein translated from the *Vupur5* cDNA failed to direct GFP to either plastids or mitochondria. The targeting sequence of glycinamide ribonucleotide transformylase (encoded by *Vupur3*) directed GFP to plastids, whereas that of AOX directed GFP to mitochondria (results not shown).

DISCUSSION

A previous study (Atkins et al., 1997) found that the complete pathway of de novo purine synthesis exists in both mitochondria and plastids isolated from cowpea nodules. Immunolocalization using AIRS-specific antiserum and Au-labeling at the EM level confirmed that the protein is present in both organelles of infected cells of the nodule central zone.

However, AIRS is apparently encoded by a single gene (Smith et al., 1998), raising the possibility that a single precursor protein may be targeted to and imported by both organelles. The subunits of the two isozymes of AIRS purified from isolated plastids and mitochondria from cowpea nodules differed in size by 0.5 kD, and N-terminal sequencing of each showed that the mitochondrial form was five amino acids longer than plastid AIRS. Two peptides recovered from a trypsin digest of the mitochondrial AIRS had identical sequences to those deduced from the *Vupur5* cDNA, consistent with this gene encoding the mitochondrial isoform.

AIRS was also recovered from both mitochondria and plastids isolated from the active meristematic tip of the primary seedling root of cowpea. Thus dual localization of the protein is not specific to nodules and is likely to occur in any other tissues of cowpea where the purine pathway is expressed. Although the two AIRS isoforms were recovered in roughly equal proportion from nodule extracts (Fig. 4), there

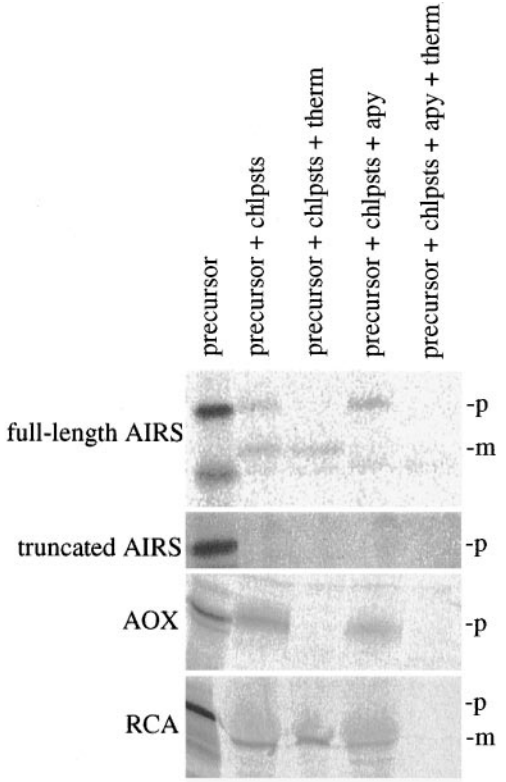

Figure 7. Pea leaf chloroplast import assay. ^{35}S-labeled precursors were incubated with purified chloroplasts (+ chlpsts lanes) and were in some cases subsequently treated with 120 μg of thermolysin (+ therm lanes). Negative control reactions were first incubated with 1 unit of apyrase (+ apyrase lanes). Precursors and mature proteins are denoted by -p and -m, respectively. The asterisk denotes the shorter translation product present in the full-length cowpea AIRS sample.

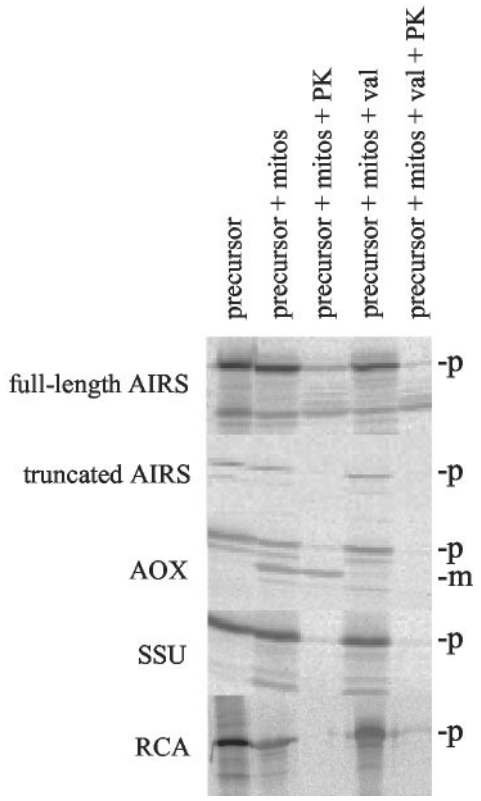

Figure 8. Mitochondrial import assay using soybean cotyledon mitochondria. Precursors were incubated with purified soybean cotyledon mitochondria (+ mitos lanes) and were in some cases subsequently treated with 3.2 μg of proteinase K (+ PK lanes). Negative control reactions contained 20 μM valinomycin (+ val lanes). Precursors and mature proteins are denoted by -p and -m, respectively.

was proportionally much more of the plastid isoform in root tip extracts (Fig. 6). There are few morphometric data for the frequency of plant organelles in nodules, but Millar et al. (1995) estimate more than 12,000 mitochondria in the infected cells of soybean. Comparable estimates for the frequency of plastids are not available, but casual observation of sections that include the cytosol at the cell periphery (e.g. Fig. 4 of Millar et al., 1995) indicates a much lower number of plastid profiles—possibly one-tenth that of mitochondria. Thus, if import into mitochondria occurs at a relatively low frequency compared with plastids, the large numbers of mitochondria present in infected nodule cells would result in significant localization to the organelle.

The AIRS protein translated from the *Vupur5* cDNA was imported into pea leaf chloroplasts by an ATP-dependent mechanism and processed to a size that corresponded to the mature protein in vivo. Pea leaf plastids are the material used routinely for in vitro assays (Bruce et al., 1994) and have been found to import and process precursor proteins targeted to a number of different plastid types. It was not possible to isolate sufficient intact plastids from cowpea nodules to carry out import assays and confirm that AIRS was also imported by these organelles. The N terminus of the isolated plastid isoform is the same as the deduced sequence of the cDNA at all but one position where a G in the deduced sequence was G or L in the isolated protein. Together, these results are consistent with the AIRS protein isolated from cowpea nodule plastids being derived from the gene represented by the *Vupur5* cDNA.

However, it appears that the transit sequence determined by N-terminal sequencing is not sufficient for import of a passenger protein (in this case GFP) into chloroplasts, suggesting that elements of the mature protein are required for its import. This was also the case for the mitochondrial presequence. Perhaps these results are a consequence of using a heterologous host, Arabidopsis, for the assays, but this seems unlikely, because in the in vitro assay, the AIRS protein was imported into pea chloroplasts.

Although the sequencing data confirm that in cowpea, AIRS is localized to mitochondria, the cowpea protein could not be imported into mitochondria isolated from a number of plant (including cowpea nodule) and non-plant sources. The region around the cleavage site of mitochondrial AIRS deduced from the N-terminal sequence does not resemble the "typical" motif for mitochondrial processing (Gavel and von Heijne, 1990), however, it has features in common with other plant mitochondrial presequences (Sjöling and Glaser, 1998) that suggest it could be recognized by the mitochondrial processing peptidase. Val is at position −3 (based on the deduced amino acid sequence of *Vupur5*) relative to the cleavage site (as in 12% of the presequences compared by Sjöling and Glaser [1998]); Ala at −2 (8%), Ala at +1 (36%), and Ser at +2 (20%).

All 11 amino acids predicted from N-terminal sequencing of the purified mitochondrial AIRS protein were identical to those deduced from the *Vupur5* cDNA, a situation that would be possible but unlikely if the protein was derived from a separate gene. Furthermore, sequence analysis based on two peptides derived from the non-terminal regions of the mitochondrial AIRS confirmed sequence identity with the *Vupur5* cDNA. Thus, despite the lack of confirmation by in vitro and in vivo import assays that the protein product from the *Vupur5* cDNA is imported into mitochondria, we conclude that the AIRS protein isolated from nodule mitochondria is likely to be derived from the gene represented by the *Vupur5* cDNA and that the same gene produces the plastid form of the enzyme.

A number of possibilities might account for the failure to demonstrate mitochondrial import of AIRS. Some proteins are only imported into mitochondria cotranslationally, for example, isoform 2 of rat liver adenylate kinase (Nobumoto et al., 1998), and this may be the case for cowpea AIRS assayed under the standard in vitro conditions used. Rapid folding of the precursor after translation may prevent post-

translational import into mitochondria. So far, attempts at in vitro cotranslational import of AIRS precursors into mitochondria have been unsuccessful. Similarly, adding the soluble extract prepared from the cytosol of infected nodule cells, to provide some "factor" missing in the in vitro assays, did not result in import or processing.

The mitochondria isolated from nodules were from relatively mature tissues, and it is possible that organelles from much younger tissues were more competent for protein import, particularly if the "window" of import competence coincided with enhanced expression of AIRS. Developmental regulation of protein import has been reported for tobacco (*Nicotiana tabacum*) leaf mitochondria (Dessi and Whelan, 1997) and for proplastids compared with mature chloroplasts in wheat (*Triticum aestivum*; Dahlin and Cline, 1991). However, nothing is known of the ontogeny of plant organelles during the differentiation of infected nodule cells. On western blots of proteins from nodules just before N_2 fixation began (from plants 14 d after sowing), the band representing mitochondrial AIRS was already visible (Fig. 4), although the fact that its intensity increased in nodules from older plants suggests that import also occurred after this stage of development. The difficulty in isolating sufficient organelles from very small nodules meant that it was not possible to test whether mitochondria isolated from nodules early in development (well before N-fixation began) were more import competent than those from the older nodules that we tested.

CONCLUSIONS

Although we have been able to find convincing data for the import of AIRS by plastids but not mitochondria using in vitro and in vivo import assays, there seems to be little doubt that the product of *Vupur5* is imported by both organelles in vivo. The two isoforms are distinguished by being cleaved to the mature organellar proteins at different sites in the targeting presequence. This phenomenon is not a nodule-specific property but occurs in other tissues where expression of the purine pathway is enhanced. Whether there are novel features associated with mitochondrial import of this class of dual-targeted protein is an important question for further research.

MATERIALS AND METHODS

Plant Material

Nodulated cowpea (*Vigna unguiculata* L. Walp cv Vita 3 inoculated with *Bradyrhizobium* sp. strain CB 756) plants were grown as described by Atkins et al. (1997). Germinating seeds for cowpea and soybean (*Glycine max*) cotyledons or pea (*Pisum sativum*) leaves were cultured as in Bruce et al. (1994), except that the tropical legumes were grown at 28°C with a 16-h daylength. Nodulated soybean (*Glycine max* [L.] Merr. cv Canopolis) plants were grown as for cowpea, except they were inoculated with *Bradyrhizobium japonicum* USDA110. Nodules were harvested from 28-d-old plants in each case. For harvest of etiolated rice (*Oryza sativa*) leaves, seeds of rice were planted in approximately 3 cm vermiculite and grown in the dark at 18°C. Plants were watered daily, and leaves were harvested after 12 d. The apical 3 to 5 mm of the primary root tip was harvested from surface-sterilized cowpea seed germinated on moist paper at 27°C in the dark for 4 d.

Immunolocalization

For immunolabeling of tissue sections for light and electron microscopy, nodules were prepared and stained according to the methods described by Fedorova et al. (1999). The rabbit polyclonal AIRS antisera have been described previously (Smith et al., 1998).

Enzyme Assays

AIRS was assayed spectrophotometrically as described by Schrimsher et al. (1986), and β-HBD and Glu dehydrogenase as described by Shelp et al. (1983) and Atkins et al. (1975), respectively. Intactness of membranes in bacteroid preparations was assessed by measuring the activity of β-HBD before and after treatment of bacteroids with Triton X-100.

Isolation of Organelles

Chloroplasts were isolated according to Bruce et al. (1994), and their chlorophyll content was assayed spectrophotometrically (Lamppa, 1995). Mitochondria from green and etiolated plant tissues were isolated as in Day et al. (1985). The same method was modified as described by Atkins et al. (1997) and was used to isolate mitochondria from cowpea and soybean nodules. Mitochondria from rat liver were isolated according to Rickwood et al. (1987). Yeast cells were grown and mitochondria was isolated using a method modified from Glick and Pon (1995).

To obtain cowpea root nodule plastids free from mitochondrial contamination, a modification of the Percoll (Amersham Biosciences AB, Uppsala) gradient centrifugation method of Journet (1987) was used. The cowpea plants were held in continuous darkness for 48 h to reduce the starch content of nodules before harvest and extraction. These plastids copurified with bacteroids. Plastid extract was separated from intact bacteroids by snap-freezing the plastid/bacteroid suspension in liquid nitrogen, followed by centrifugation to remove intact bacteroids and plastid membranes.

Plastids and mitochondria were separated and isolated from 15 to 23 g fresh weight of primary root tips (3–5 mm) collected from 4-d-old cowpea seedlings using the same methods as above (Journet, 1987) for nodule organelles. This method provides optimal separation of plastids and mitochondria by centrifuging the intact organelles over a 35% (v/v) Percoll solution in a swinging bucket rotor. Under these conditions, a gradient of Percoll does not form, and mitochondria collect at the top in the interface between the soluble extract and the Percoll solution while plastids migrate to the bottom of the centrifuge tube and collect as a pellet. After collection, the two organelle fractions were washed twice in buffered (pH 7.2) 0.3 M Suc wash solution containing 0.1% (w/v) bovine serum albumin. The protein in the isolated and washed organelle fractions was collected as an acetone precipitate (−80°C) and solubilized in SDS running buffer before SDS-PAGE.

PAGE

Discontinuous Tris-Gly SDS-PAGE was as described by Laemmli (1970), and discontinuous native PAGE as described by Gallagher (1995). Proteins were visualized directly on gels by staining with Coomassie Brilliant Blue R250 (Sigma-Aldrich, St. Louis) or by silver staining (Merril et al., 1984). Gels were alternatively electroblotted onto nitrocellulose (Protran) or polyvinylidene difluoride (Problott) membranes according to Towbin et al. (1979) and Ursitti et al. (1995), respectively. Proteins were visualized on nitrocellulose membranes by immunostaining using polyclonal antisera raised to an AIRS recombinant protein (Smith et al., 1998) or on polyvinylidene difluoride membranes by staining with Coomassie Brilliant Blue R250 (Ursitti et al., 1995).

Two-Dimensional Gel Electrophoresis and MS/MS Peptide Analysis

Mitochondria were prepared from 40 to 50 g fresh weight of cowpea nodules as described above, and the proteins were extracted and precipi-

tated in acetone (−80°C). Isoelectric focusing separation on pH 3 to 10 nonlinear immobilized pH gradient strips (Immobiline DryStrips, APBiotech, Sydney) and second dimension SDS-PAGE were performed according to Millar et al. (2001). After two-dimensional gel electrophoresis, the position of AIRS was established by western blotting as above, and replicate gels were used to locate the AIRS protein after colloidal Coomassie (G250) staining. Spots were excised, destained, trypsin digested, and analyzed using a Q-STAR Pulsar mass spectrometer (Q-TOF, Applied Biosystems, Sydney) according to Sweetlove et al. (2001). Mass spectra and collision MS/MS data were analyzed with BioAnalyst software (Applied Biosystems).

Immunoaffinity Purification of AIRS from Cowpea Nodule Mitochondria and Plastids

Polyclonal rabbit anti-AIRS immunoglobulins were purified from total antiserum using a recombinant AIRS:cyanogen bromide Sepharose affinity resin, based on the procedure of Springer (1996). Hexa-His-tagged AIRS was affinity purified from overexpressing *Escherichia coli* cells using the QIAexpress system (Qiagen, Clifton Hill, Australia) as described by Smith et al. (1998) and coupled to pre-activated cyanogen bromide:Sepharose 4B (Pharmacia, Uppsala) by a modification of the method of Springer (1996)). Pure antibodies were immobilized on Protein A:Sepharose CL-4B (Sigma-Aldrich) and chemically cross-linked to the matrix using a combination of procedures from Hermanson et al. (1992) and Harlow and Lane (1988) and used to purify cowpea AIRS from isolated nodule mitochondria and plastids. Pure AIRS was eluted with low-pH buffer (Springer, 1996) and either immediately used for activity assays or desalted into water using a Sephadex G-50 column (Pharmacia) and freeze-dried for storage before subsequent analysis.

In Vitro Protein Import Assays

[^{35}S]Met-labeled (Amersham Biosciences) precursors for cowpea and Arabidopsis AIRS together with a truncated AIRS precursor that had the putative targeting presequence removed were translated in a TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI). The presequence was identified as the sequence where homology with the *E. coli* sequence begins (Smith et al., 1998). Chloroplast import assays were set up as in Bruce et al. (1994), with negative controls containing 1 unit of apyrase (Henry et al., 1994). Mitochondrial import assays were as described by Whelan et al. (1996), with negative controls containing 20 μM valinomycin. Each import assay was repeated at least seven times. Authentic ^{35}S-labeled mitochondrial (soybean AOX; Whelan et al., 1993; approximately 38 kD) and chloroplast (spinach RCA; Werneke et al., 1988) precursor proteins were used as positive and negative controls for import into isolated mitochondria and plastids, respectively. In all cases, results were visualized by SDS-PAGE followed by exposure of dried gels to an imaging plate (BAS-MP 2040, Fujifilm, Tokyo). Forty percent of the translation mixture was loaded onto each gel.

In Vivo Import Assays

The regions of the *Vupur5*, *Vupur3* (encoding glycinamide ribonucleotide transformylase), and AOX cDNAs encoding the targeting sequences were inserted in-frame at the 5′ end of the *mgfp5* gene in the binary vector pCAMBIA1302 (CAMBIA, Canberra, Australia). For *pur5*, this region encodes the first 71 amino acids and includes the N termini of the processed mitochondrial and plastid forms of AIRS as determined in this paper. Expression of the gene fusions was driven by a single cauliflower mosaic virus 35S promoter.

DNA from the constructs was bombarded into Arabidopsis leaves using a PDS-1000-He System (Bio-Rad, Hercules, CA), and after 24 to 48 h, the fluorescence of transiently expressed GFP was viewed using a confocal scanner TCS SPII with a DM IRBE microscope (Leica, Heidelberg) equipped with a 20× water immersion objective. Leica software was used for image analysis. GFP was excited at 488 nm using an argon laser, and fluorescence was detected in the range between 505 and 520 nm. Chlorophyll autofluorescence was detected in the window between 600 and 680 nm.

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