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Evidence for a link between translocation and processing during protein import into soybean mitochondria

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Abstract

The effect of metal chelators on protein import was investigated using isolated soybean mitochondria and soybean precursor proteins. Adding 1,10-phenanthroline, a metal chelator that can cross both mitochondrial membranes abolished import of both the alternative oxidase, and the F_Ad subunit of the ATP synthase, a matrix located protein. Other metal chelators such as EDTA, 1,7-phenanthroline and 4,7-phenanthroline, which cannot cross the mitochondrial membranes, had no effect on import. When processing, a known metal-dependent step inside mitochondria, was inhibited using a mutagenesis approach (changing a −2 arginine to a −2 glycine in the pre-piece of the precursor), so was import. Thus it would appear that in soybean, at least, translocation of proteins across the mitochondrial membrane, as well as processing, relies on a metal dependent step. Taken together, the data suggest that the two processes may be directly connected in these mitochondria.

Keywords: Mitochondrial import; Processing; Inhibiting import; (*Glycine max* L.)

1. Introduction

The translocation of nuclear encoded proteins into the mitochondrion is a multi-step process involving several protein components in both the cytosol and mitochondrion of the cell. Precursor proteins synthesised in the cytosol bind several factors which keep the precursor in an import competent state [1,2]. The precursor binds to the mitochondria via proteinaceous receptors and translocation into the mitochondria is usually dependent on both a membrane potential and ATP [3]. ISP 42 from yeast and MOM 38, its counterpart in *Neurospora crassa*, seem to be a focus for import for the majority of proteins studied to date [2,4]. It has been proposed that the bound proteins are pulled into the mitochondrion via a ratchet mechanism [5]. Once inside the matrix, proteins may undergo processing, assembly and further sorting to the correct intraorganelle compartment [2,3]. Sorting may take place by either the stop-transfer mechanism or conservative sorting depending on the protein involved [6,7].

As the mitochondrial import process is multi-step, there are several places in the import route where it can be interrupted. Import can be inhibited by treating mitochondria with externally added protease, which removes surface exposed mitochondrial receptors [3]. A more complicated method of inhibiting translocation involves trapping the protein when it is bound to the surface receptor. This can be achieved by lowering ATP levels or temperature. So called 'translocation contact site intermediates' can be generated by this means and these intermediates have proved valuable in identifying components of the import apparatus [7,8]. Import can also be inhibited by adding compounds that stabilise the tertiary structure of the protein and prevent unfolding. Binding of antibodies to the carboxyl terminus of the protein or attachment of methotrexate with dihydrofolate reductase as a passenger, is commonly used in this case [9,10]. Finally translocation can be prevented by adding compounds that dissipate the membrane potential [3].

Other steps in the import process can be disrupted without inhibiting import per se (translocation). An example of this is the re-translocation of proteins from the matrix to the inner membrane in conservative sorting [8,10]. In yeast and mammals, translocation and processing are distinct and separate processes which can be inhibited independently. For example, inhibition of processing with

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metal chelators and site-directed mutagenesis does not inhibit translocation. The precursor protein is imported under such circumstances even though it is not processed to the mature form [3,11,12].

The peptidase involved in processing effects the removal of the prepiece which directs the imported protein to the mitochondrion. This enzyme has been studied extensively from a range of organisms. It was first characterised from *Neurospora crassa* and yeast where it was shown to be a dimer of two dissimilar subunits, now called α-MPP and β-MPP [3]. β-MPP was shown to be identical to a core subunit of the cytochrome bc_1 complex in *N. crassa*. Thus this protein has two locations in *N. crassa*, the bc_1 complex and the matrix. β-MPP is encoded by a single gene in *N. crassa* [13].

In yeast and mammals, MPP is located in the matrix and has a dimer structure [14,15]. In yeast it is encoded by separate genes from those of the bc_1 complex. These genes are believed to be related to the core subunits of the bc_1 complex and to have arisen from a common ancestor [16]. The genes for MPP subunits have been isolated from a mammalian organism and shown to contain homology to those from yeast and *N. crassa*. [17,18].

In the plant species examined so far MPP is located in the cytochrome bc_1 complex and cannot be dissociated from it without loss of activity [19–21]. Thus in plants the principle processing activity appears to be located in the membrane as opposed to the matrix. Therefore the MPP of plants differs from that of all other organisms studied to date. It should be noted that other peptidases are also involved in precursor maturation in mitochondria. These peptidases act subsequently to MPP and are considered to be unrelated to MPP in origin [3].

The location of MPP in the inner membrane of plant mitochondria has led to the proposal that this situation predates that in fungi and yeast. It has been proposed that MPP belongs to a family of endopeptidases including *Escherichia coli* pitrilysin. An endopeptidase in this family was incorporated into the cytochrome bc_1 complex and thus in plants the primitive situation is found. In *N. crassa* the peptidase and the cytochrome bc_1 complex have been dissociated but the genes that code for them still overlap. In yeast and mammals, the peptidase and the genes that encode it appear to have completely separated. These differences may reflect the different lifestyles of various organisms. In yeast, a facultative anaerobe, it may be necessary to express components of the import machinery without expressing components of the respiratory chain, and this is reflected by the fact that in yeast MPP is encoded by separate genes from those of the cytochrome bc_1 complex [16,22].

Fungal mitochondrial proteins can be imported into plants and animals, plant proteins imported into yeast, and mammalian precursors imported into plants [3,23–25]. This has led to the belief that the import process is universal and characterisation in a few organisms will give a complete understanding of mitochondrial protein targeting. However, in plants, where at least one component of the import machinery differs in location to that in other organisms, the import process may be different. In particular, the location of the processing enzyme MPP in the membrane bound bc_1 complex may indicate a closer linkage between translocation and processing. With this in mind, we have re-investigated the effect of inhibiting processing on protein import, using soybean cotyledon mitochondria as a model system.

2. Materials and methods

2.1. Mitochondrial import

^{35}S-labelled precursor proteins for soybean alternative oxidase [26] and the F_Ad subunit of the ATP synthase [27] were synthesised in a T_NT coupled transcription translation system from Promega (Madison). Both genes were transcribed using T7 RNA polymerase and translated in a rabbit reticulocyte lysate system. Mitochondria were isolated from 7 day old soybean cotyledons according to Day et al. [28] and import was carried out according to Whelan et al. [29] using succinate as a substrate [30]. Each import reaction consisted of 100 μg of mitochondrial protein in 200 μl of import buffer previously described [29]. The precursor protein was then added and the reaction commenced by warming to room temperature (23°C). Imports were allowed to proceed for 20 min and then divided into two 100 μl aliquots and placed on ice. One 100 μl aliquot of each import reaction was treated with Proteinase K (PK) to a final concentration of 2 μg/ml. After PK treatment, PMSF was added to 2 mM to both aliquots and mitochondria were sedimented by centrifugation, lysed in gel loading buffer and products analysed by SDS-PAGE and autoradiography using standard procedures [31].

The import efficiency was determined by comparing the amount of imported product (PK protected product) to the amount of precursor added. Import was expressed as a percentile value, the amount of precursor added representing 100%. In the case of the inhibitor titration, the effect of the inhibitor was compared to the reaction without inhibitor. Thus 100% import represents import in the absence of inhibitor and the other values are relative to this degree of import. Quantitation of import reactions was carried out by scanning the autoradiographs and analysing the bands using NIH image. The presequence of the alternative oxidase is predicted to contain 6 of the 16 methionine residues in the total protein [26]. These 6 methionine residues are removed upon processing to the mature form. Therefore the % import would apparently be underestimated by scanning the intensity of the mature bands alone. However, 5 out of 6 of the methionine in the predicted presequence are at the start of the protein and radiosequencing confirmed the location of one methionine, not

five (Whelan and Day, unpublished data). Thus the % import values are not corrected for the removal of the presequence but represent the intensity of the bands shown. (However, adjustment of the % import for 6 or 2 methionine residues in the removed presequence gave similar differences when compared between samples (data not shown)).

2.2. Addition of inhibitors

The inhibitors used were made up at 100 times the final concentration. In the case of 1,10-phenanthroline, 1,7-phenanthroline and 4,7-phenanthroline the stocks were made up in 100% ethanol. In the case of EDTA the stock was made up in sterile distilled water. The inhibitors were added to mitochondria on ice and allowed to incubate for 3 minutes before the addition of precursor and warming to room temperature. Controls had a similar amount of EtOH added.

2.3. Mutagenesis

Comparison of the transit peptides of several alternative oxidase proteins from different plant species showed that they all contained a −2 arginine residue before the beginning of the mature protein as determined by N-terminal sequencing by Rhoads and McIntosh [32] (Fig. 1, top) [32]. The −2 arginine has been proposed as a processing signal in mitochondria and such a mutant (arginine to glycine) in the soybean alternative oxidase precursor was not processed by mitochondrial extracts from soybean (Fig. 1, bottom) [30,33,34]. Thus we used this precursor to compare its import to the normal precursor. We also created additional mutants where we changed the −10 arginine residue to a glycine, and the −2 arginine residue to lysine (Fig. 1, bottom). Site directed mutagenesis was carried using an altered site mutagenesis kit from Promega. Mutants were confirmed by sequencing using an ABI 370A sequencer with dye terminators using the T7 sequencing primer according to the manufacturers instructions. The oligonucleotides used to create the mutants were:

−2 Mutant (Arg-Gly)	5′ GTGGTGTGGGGAGTGAGAGC 3′
−2 Mutant (Arg-Lys)	5′ GTGGTGTGAAGAGTGAGAGC 3′
−10 Mutant (Arg-Gly)	5′ GGTGGTTTAGGAGCATTATATGG 3′

These oligonucleotides changed the bases (underlined) to convert the residues as outlined in Fig. 1, bottom [30].

3. Results

3.1. Addition of metal chelators

The in vitro synthesised precursor of the alternative oxidase from soybean was efficiently imported into isolated soybean mitochondria and processed to a mature form (Fig. 2, lanes 2 and 3) [30]. The precursor form (P) of 36 kDa was converted to the mature form (M) of 32 kDa. Greater than 95% of the protease protected protein was mature form as judged by image analysis of 20 separate import reactions (data not shown). The effect of adding various metal chelators (characteristic inhibitors of processing in fungal and mammalian systems) to the import reaction is shown in Fig. 2. Addition of EDTA to 2 mM had no effect on import or processing (Fig. 2, lanes 4 and 5). However, addition of 1,10-phenanthroline to 1 mM inhibited import 8 fold. In the presence of 1,10-phenanthroline only 5% of added precursor was imported into the mitochondrion as judged by the sensitivity of the added precursor to externally added PK (Fig. 2, lanes 6 and 7). It should be noted that 5% import represents a maximum and is the highest level we have seen in several such experiments. Typically it is lower (approx. 1–2%) and cannot be detected under normal exposure conditions. This is evident

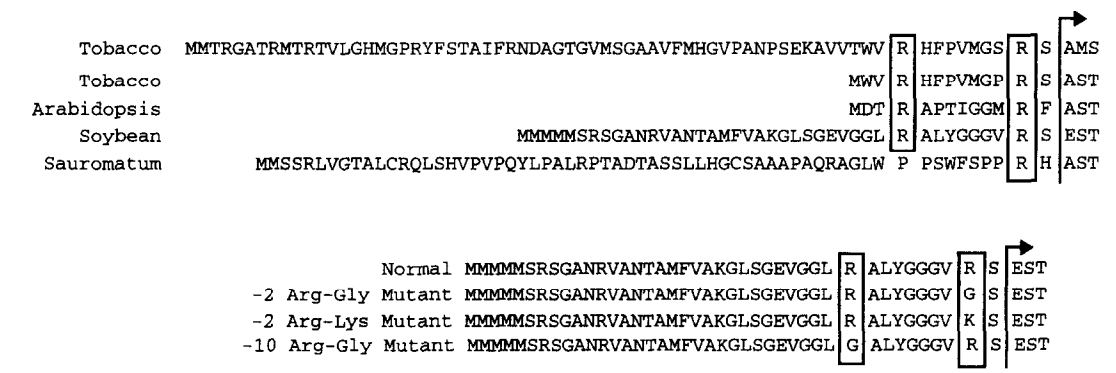

Fig. 1. Transit peptides of all alternative oxidase precursors. (Top) The transit peptide of the alternative oxidase from four plant species. Two sequences are shown for tobacco as two separate gene sequences have been reported. The start of the mature protein is indicated by the arrow and the conserved arginine residues are boxed. (Bottom) The soybean alternative oxidase pre-piece of the normal and mutated precursors. The changed residues are boxed. The start of the mature protein is shown by an arrow.

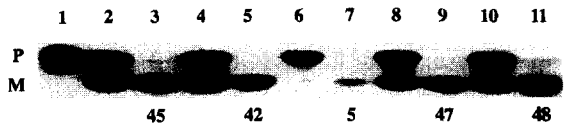

Fig. 2. Import of the soybean alternative oxidase into mitochondria in the presence of various inhibitors. The figures underneath represent the % import compared to the amount of precursor added to the import reaction. Lane 1, precursor protein alone, this represents 40% of the precursor added to each import reaction. Lane 2, import without any inhibitor added. Lane 3, as lane 2 with PK added. Lane 4, as lane 2 with EDTA added to 2 mM before the precursor protein. Lane 5, as lane 4 with PK added. Lane 6, as lane 2 in the presence of 2 mM 1,10-phenanthroline. Lane 7, as lane 6 with PK added. Lane 8, as lane 2 in the presence of 2 mM 1,7-phenanthroline. Lane 9, as lane 8 with PK added. Lane 10, as lane 2 with 4,7-phenanthroline added to 2 mM. Lane 11, as lane 10 with PK added.

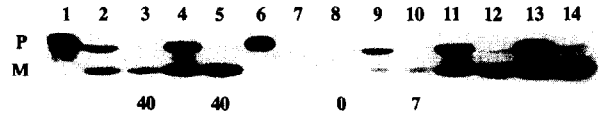

Fig. 4. Import of normal alternative oxidase and the −2 Arg-Gly mutant. The numbers underneath represent the % import compared to the amount of precursor added to the import reaction. Lane 1, normal precursor only (2 μl of translation lysate). Lane 2, import of normal protein (2.5 μl of translation lysate). Lane 3, as lane 2 with PK added. Lane 4, as lane 2 with 5 μl of translation lysate. Lane 5, as lane 4 with PK added. Lanes 6, 7, 8, 9, 10, as lanes 1 to 5, respectively, except that the −2 mutant alternative oxidase was used. Lane 11, import of 5 μl of normal alternative oxidase in the presence of 2.5 μl translation lysate of the −2 mutant. Lane 12, as lane 11 with PK added. Lane 13, as lane 11 in the presence of 5 μl of the −2 mutant translation lysate. Lane 14, as lane 13 with PK added.

in Fig. 3, lanes 4 to 7 where import of the alternative oxidase in the presence of 1 and 2 mM 1,10-phenanthroline was undetectable. In contrast the 1,7 and 4,7 forms of phenanthroline, which cannot cross the mitochondrial inner membrane had no effect on import (Fig. 2, lanes 8 to 11) [3]. The various forms of phenanthroline and EDTA have no effect on oxygen consumption and coupling up to 5 mM concentration (data not shown). The inhibition by 1,10-phenanthroline is in contrast to fungal systems where addition of this mitochondrial permeable metal chelator, a characteristic inhibitor of MPP, does not inhibit import. However, the imported product in the fungal system is largely the precursor form [11].

To further investigate this unexpected inhibition of import we carried out dilutions with 1,10-phenanthroline to determine at what concentration inhibition was effective (Fig. 3). 50% inhibition of import (as judged by the amount of PK protected protein) was achieved at 0.25 mM 1,10-phenanthroline; greater than 95% inhibition was consistently achieved with 1 mM (Fig. 3). In this experiment we could not detect import with 1 mM 1,10-phenanthroline. In fungal systems 1 mM 1,10-phenanthroline has no effect on import [11,12]. In plants higher amounts (> 5 mM) are frequently used with isolated processing peptidase to inhibit processing although we have found 2 mM to be effective [35,36]. We tested the effect of EDTA and all forms of phenanthroline on oxygen uptake up to concentration of 5 mM. No effect on oxygen uptake or coupling was apparent (data not shown). As 1,10-phenanthroline is widely used in other systems to inhibit processing without inhibiting import and has no apparent effect on oxygen uptake in our system (or other systems), we conclude that there is a metal-dependent translocation step on the inside of mitochondria.

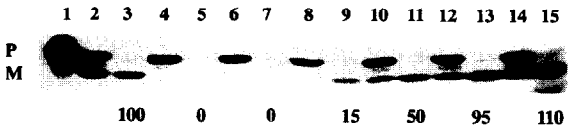

Fig. 3. Import of the soybean alternative oxidase in the presence of various concentrations of 1,10-phenanthroline. 100% import is defined as the amount of import in the absence of any inhibitor. All other values are relative to this value. Lane 1, precursor protein alone. Lane 2, import with no inhibitor added. Lane 3, as lane 2 with PK added. Lanes 4, 6, 8, 10, 12 14, 1,10-phenanthroline added to 2, 1, 0.5, 0.25, 0.125 and 0.0625 mM, respectively. Lanes 5, 7, 9, 11, 13, 15 as lanes 4, 6, 8, 10, 12, 14, respectively, with PK added.

3.2. Inhibition of processing using site-directed mutagenesis

Proteolytic processing is one of the best characterised metal dependent steps in the import apparatus [3,22,35,37]. We therefore attempted to inhibit processing to observe the effect on mitochondrial import. Previously we had created a mutant of the soybean alternative oxidase precursor which was not processed by mitochondrial extracts in processing assays [30]. This mutant involved the conversion of an arginine residue at position −2 to the mature protein to a glycine residue. Such a change had previously been documented to inhibit processing in mammalian systems [38]. We also created a number of other mutations and tested their effect on import. We carried out import experiments on the normal alternative oxidase, the −2 Arg-Gly, −2 Arg-Lys and the −10 Arg-Gly mutant. The results are shown in Figs. 4 and 5.

Each precursor was incubated with mitochondria and

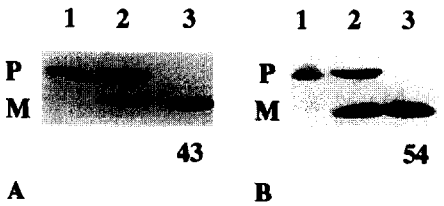

Fig. 5. Import of the −10 Arg-Gly mutant (A) and −2 Arg-Lys mutant (B) into isolated mitochondria. The figures underneath represent the % import compared to the amount of precursor protein added. (A) Lane 1, precursor alone (P). Lane 2, precursor with mitochondria, an additional mature form (M) is evident. Lane 3, as lane 2 with PK added, only the mature form is evident. (B) As panel A except that the −2 Arg-Lys mutant precursor was used.

the efficiency of import determined by comparing the amount of the imported mature, PK-protected product to the amount of precursor protein added. As is typically seen with the alternative oxidase precursor, 43% of the added precursor was imported. Import was carried out with two different amounts of added precursor to account for any variation in translation efficiency that existed between the different constructs. Using different amounts of the alternative oxidase precursor protein, 40% of the added precursor was imported (Fig. 4, lanes 1 to 5). In contrast, import of the −2 Arg-Gly mutant was severely inhibited. Only 7% of the added precursor was imported, and this could only be detected when a higher amount of precursor was used (Fig. 4, lanes 6 to 10). The translation efficiency of the normal alternative oxidase and −2 Arg-Gly mutant differed, the latter being 70% of the intensity of the former (data not shown). However, we have used a range of concentrations and import levels are always low with the −2 Arg-Gly mutant and could only be detected with higher amounts of the precursor protein. It is important to note that for the −2 Arg-Gly mutant, the unimported protein was present in the supernatant of the import reaction. However, it did not pellet with the mitochondria during re-isolation after the import reaction and treatment. This indicated that the −2 Arg-Gly mutant bound to the mitochondria less tightly and was removed upon separation of the mitochondria (Fig. 4). Inclusion of the −2 Arg-Gly precursor in the reaction mixture containing the normal precursor did not effect import of the latter (Fig. 4, lanes 11 to 15). We have not shown import efficiencies for the alternative oxidase in the presence of the −2 Arg-Gly mutant. However, it is apparent that import is as efficient or more so than the normal alternative oxidase alone. This slightly higher import efficiency may be due to the presence of more rabbit reticulocyte lysate which will contain factors that support import.

Import efficiency for the −10 Arg-Gly mutant was similar to the normal alternative oxidase, with 40% of the precursor being imported (Fig. 5A). Other mutations of the −2 Arg did not inhibit import. In fact import of the −2 Arg-Lys mutant was more efficient, with greater than 50% of the precursor being imported (Fig. 5B). The translation efficiency of this precursor was 50% of the normal alternative oxidase, lower than the −2 Arg-Gly mutant, yet, import proceeded efficiently. This indicates that import efficiency was not dependent on the translation efficiency of the precursor. Another −2 mutant (−2 Arg-Leu) was also imported normally even though its translation efficiency was only 20% that of the normal alternative oxidase (data not shown). As can be seen in Fig. 5 these mutants are processed to give a correct size mature product. From these results it appears that only a mutation that inhibits processing also inhibits import.

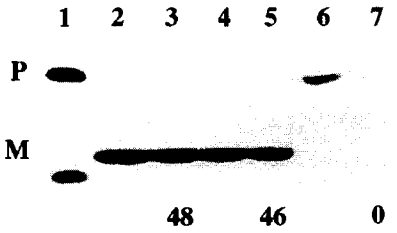

Fig. 6. Import of the precursor to the F_Ad subunit of the ATP synthase in the presence of inhibitors. The figures underneath represent the % import compared to the amount of precursor added. Lane 1, precursor alone, this represents 40% of the precursor added to the import reaction. Lane 2, import of F_Ad precursor into mitochondria. Lane 3, as lane 3 with PK added. Lane 4, as lane 2 with EDTA added to 2 mM. Lane 5, as lane 4 with PK added. Lane 6, as lane 2 with 1, 10 phenanthroline added to 2 mM. Lane 7, as lane 6 with PK added.

3.3. Import of a matrix-located protein in the presence of inhibitor

The above studies utilised the alternative oxidase, an inner membrane protein. Similar experiments were carried out on a matrix-located protein, the F_Ad subunit of the mitochondrial ATP synthase from soybean [27]. Synthesis of this protein in an in vitro coupled translation system led to a precursor (P) with an apparent mol mass of 24 kDa (Fig. 6, lane 1), which upon import was converted to a mature form of 21 kDa (Fig. 6, lanes 2 and 3). Import of this precursor proceeded normally in the presence of 2 mM EDTA but is severely inhibited in the presence of 2 mM 1,10-phenanthroline (Fig. 6, lanes 4 to 7). In fact, we could not detect import of the F_Ad subunit in the presence of 1,10-phenanthroline. Thus the import of a matrix-located protein is also dependent on a metal catalysed step on the inside of the mitochondrion.

4. Discussion

We have shown that the addition of 1,10-phenanthroline inhibits protein import into soybean mitochondria. This was shown for the alternative oxidase, an inner membrane protein and the F_Ad subunit of the mitochondrial ATP synthase, a matrix located protein. 1,10-phenanthroline, a metal chelator is a characteristic inhibitor of the general mitochondrial processing peptidase (MPP) from a variety of organisms. In contrast we could not inhibit import with intact mitochondria using EDTA, another effective, but impermeant inhibitor of MPP [35]. This was confirmed using 1,7- and 4,7-phenanthroline which also cannot cross the inner mitochondrial membrane. Thus we conclude that the metal dependent import step is on the inside of the mitochondrial inner membrane [3].

When similar studies using inhibitors are carried out in fungal systems no inhibition of import is detected [12,39,40]. In these studies EDTA was commonly used in addition to 1,10-phenanthroline to inhibit processing. The logic has been that EDTA binds excess ions outside the mitochondrion that would otherwise bind the 1,10-phenan-

throline. We do not find it necessary to add EDTA to inhibit processing with intact plant mitochondria. As EDTA, 1,7-phenanthroline and 4,7-phenanthroline have no effect on import at 2 mM, the effect of the 1,10-phenanthroline on import cannot be accounted for by simply an effect of chelating metal ions outside the mitochondrion. Finally, the effective concentration of 1,10-phenanthroline used (1–2 mM) has been used in other systems without inhibiting import.

In order to investigate the nature of the metal dependent import step, we carried out site-directed mutagenesis to inhibit processing, a well characterised metal dependent step in the import process from a variety of organisms [3,37]. The use of site-directed mutagenesis to inhibit processing has been detailed previously. For example, in the rat, alteration of the precursor to ornithine transcarbomylase by changing an arginine at position 23, to a glycine residue, inhibits processing but not import [38,41]. This precursor is processed in two parts and residue 23 is considered to be the −2 arginine signal [38,41]. In our case change of the arginine to glycine inhibits both processing and import. We know of only one other report where change of an arginine to a glycine inhibits both processing and import. This was reported for human ornithine transcarbomylase where change of the arginine 23 to glycine resulted in inhibition of import (into rat liver mitochondria) as well as processing [42]. This appears in conflict with the report above where such a change on the rat precursor had no effect on import into rat mitochondria. This anomaly may be explained by the fact that a completely homologous system was not used. We, on the other hand, have used a completely homologous system and do not believe that this inhibition of import is due to the disruption of the α-helical nature of the presequence, as suggested in [42], for several reasons. Firstly, substitution of a glycine for arginine at −10 has no effect on processing and import in our system, and such a similar substitution would be expected to disrupt the α-helical nature of the presequence (if indeed it is α-helical — see below). Likewise this substitution has the same charge effect on the transit peptide, so an overall change in charge cannot account for the inhibition by the substitution of glycine for arginine at −2. Secondly, examination of the soybean presequence (Fig. 1, top) shows that there are three glycine residues at positions −4 to −7 of the soybean alternative oxidase presequence. Thus the presequence is unlikely to be α-helical in this region and this is confirmed by computer predictions (data not shown). Finally, analysis of mitochondrial presequence structure indicates that the last 10 to 15 amino acids are not generally helix forming [43]. Our analysis of the soybean transit peptide has confirmed this and we suggest that the inhibition of import seen with the −2 Arg-Gly mutant was not the result of disturbing the helical nature of the presequence.

We have shown that there is a metal dependent import step inside mitochondria in soybean mitochondria. Site directed mutagenesis to change a single amino acid residue inhibited both processing and translocation. These results are consistent with the possibility that processing and translocation of proteins are closely linked in soybean mitochondria such that inhibiting processing inhibits translocation. However, it is obvious that processing is not absolutely essential for all proteins imported into plant mitochondria since several nuclear encoded proteins are synthesised without cleavable presequences in plants and are still imported [44–47]. Rather we suggest that if a precursor has a cleavable prepiece and removal of this prepiece is prevented, then import is inhibited. This may suggest a close association of MPP and the translocation machinery in plants.

In this context it should be noted that the adenine nucleotide translocator is synthesised with a cleavable prepiece in plants [48]. If this prepiece is removed the protein can be imported into mitochondria without processing, the targeting information being in the mature part of the protein as is the case for the ANT from other organisms [48]. It has been reported that the cleavable prepiece of the ANT from potato cannot target passenger proteins to the mitochondrion [50]. However, if the ANT protein contains only some residues of this cleavable prepiece (not involved in targeting), and 1,10-phenanthroline is included in the import reaction, import is inhibited [49]. This indicates that 1,10-phenanthroline is likely to inhibit the import of all proteins into plant mitochondria, not only proteins that contain the targeting information in a cleavable prepiece. This indicates that the processing machinery may be physically close to the translocation apparatus and that inhibiting the former causes a conformation that is incompatible with translocation. However, it does not indicate that processing is essential for import, even for proteins that contains a cleavable prepiece.

The results presented show that two processes previously documented to inhibit processing in plant mitochondria also inhibit translocation. This may indicate that there is a close association between processing and translocation in soybean mitochondria. As processing takes place in the matrix some translocation of the protein has to take place before processing is possible. Inhibition of processing while the precursor is still being translocated with 1,10-phenanthroline may result in steric interference with the translocation process but the precursor protein still remains bound to the mitochondrion. The precursor still has the binding affinity for the processing enzyme but the enzyme cannot undergo the catalytic mechanism. In contrast inhibition of processing with site directed mutagenesis probably results in an lower binding affinity of the precursor for the processing enzyme. A decrease in binding affinity of a peptide to MPP has been reported when an arginine residue has been changed [51]. As the precursor has been transferred to the translocation machinery but cannot be translocated because processing has been inhibited it will not stay associated with the mitochondria

during subsequent isolation and thus display a lower binding affinity.

In summary, our results suggest that there may be a close physical association between the import and processing machinery in plant mitochondria. Inhibition of import by 1,10-phenanthroline and subsequent analysis of the structure may lead to the identification of components of the import machinery in plants.

References

[1] Murakami, H., Pain, D. and Blobel, G. (1988) J. Cell Biol. 107, 2051–2057.
[2] Glick, B. and Schatz, G. (1991) Annu. Rev. Genet. 25, 21–44.
[3] Hartl, F-U., Pfanner, N., Nicholson, D.W. and Neupert W. (1989) Biochim. Biophys. Acta 988, 1–45.
[4] Baker, K.P., Schaniel, A., Vestweber, D. and Schatz, G. (1990) Nature 348, 650–608.
[5] Schneider, H.C., Berthold, J., Bauer, H.F., Dietmeier, K., Guiard, B., Brunner, M. and Neupert, W. (1994) Nature 371, 768–774.
[6] Glick, B.S., Beasley, E.M. and Schatz, G. (1992) Trends Biochem. Sci. 17, 453–459.
[7] Shore, G.C., Millar, D.G. and Li, J-M (1992) in Membrane Biogenesis and Protein Targeting (Neupert, W. and Lill, R., eds.), pp. 253–264, Elsevier Science.
[8] Beasley, E.M., Wachter, C. and Schatz, G. (1992) Curr. Opin. Cell Biol. 4, 646–651.
[9] Schwaiger, M., Herzog, V. and Neupert, W. (1987) J. Cell Biol. 105, 235–246.
[10] Cheng, M.Y., Hartl, F.U., Martain, J., Pollock, R.A., Kalousek, F., Neupert, W., Halberd, E.M., Halberd, R.L. and Horwich, A.L. (1989) Nature 337, 620–625.
[11] Schneider, H., Arretz, M., Wacter, E. and Neupert, W. (1990) J. Biol. Chem. 265, 9881–9887.
[12] Japa, S. and Beattie, D.S. (1994) Arch. Biochem. Biophys. 312, 414–420.
[13] Weiss, H., Leonard, K. and Neupert, W. (1990) Trends Biochem. Sci. 15, 178–180.
[14] Saavedra-Alanis, V.M., Rysavy, P., Rosenberg, L.E. and Kalousek, F. (1994) J. Biol. Chem. 269, 9284–9288.
[15] Witte, C., Jensen, R.E., Yaffe, M.P. and Schatz, G. (1988) EMBO J. 7, 1439–1447.
[16] Schulte, U., Arretz, M., Schneider, H., Tropschug, M., Wachter, E., Neupert, W. and Weiss, H. (1989) Nature 339, 147–149.
[17] Kitada, S., Niidome, T., Nagano, T., Ogishima, T. and Ito, A. (1993) Biochem. Biophys. Res. Commun. 190, 289–293.
[18] Paces, V., Rosenberg, L.E., Fenton, W.A. and Kalousek, F. (1993) Proc. Natl. Acad. Sci. USA 90, 5355–5358.
[19] Eriksson, AC. and Glaser E. (1992) Biochim. Biophys. Acta 1140, 208–214.
[20] Eriksson, A.C., Sjoling, S. and Glaser, E. (1994) Biochim. Biophys. Acta, 1186, 221–231.
[21] Emmermann, M., Braun, H-P, Arretz, M. and Schmitz, U.K. (1993) J. Biol. Chem. 268, 18936–18942.
[22] Braun, H-P. and Schmitz, U.K. (1995) Trends Biochem. Sci. 20, 171–175.
[23] Chaumont, F. and Boutry, M. (1995) in The Molecular Biology of Plant Mitochondria, (Levings, III, C.S. and Veril, I.K., eds.), Kluwer, Dordrecht.
[24] Whelan, J., Dolan, L. and Harmey, M.A. (1988) FEBS Lett. 236, 217–220.
[25] Luzikov, V.N., Novikova, L.A., Whelan, J., Hugosson, M. and Glaser, E. (1994) Biochem. Biophys. Res. Commun. 199, 33–36.
[26] Whelan, J., McIntosh, L. and Day, D.A. (1993) Plant Physiol. 103, 1481.
[27] Smith, M.K., Day, D.A. and Whelan, J. (1994) Arch. Biochem. Biophys. 313, 235–240.
[29] Whelan, J., Knorpp, C. and Glaser, E. (1990) Plant Mol. Biol. 14, 977–982.
[28] Day, D.A., Neuberger, M. and Douce, R. (1985) Aust. J. Plant Physiol. 12, 119–130.
[30] Whelan, J., Hugosson, M., Glaser, E. and Day, D.A. (1995) Plant Mol. Biol. 27, 769–778.
[31] Wienhaus, U., Koll, H., Becker, K., Guiard, B., Hartl, F-U. (1992) in Protein Targeting, A Practical Approach, (Magee, A.I. and Wileman, T., eds.), pp. 135–159, IRL Press, Oxford
[32] Rhoads, D.M. and McIntosh, L. (1991) Proc. Natl. Acad. Sci. USA 88, 2122–2126.
[33] Hendrick, J.P., Hodges, P.E., Rosenberg, L.E. (1989) Proc. Natl. Acad. Sci. USA 86, 4056–4060.
[34] Von Heijne, G., Steppuhn, J. and Herrmann, R.G. (1989) Eur. J. Biochem. 180, 535–545.
[35] Emmermann, M. and Schmitz, U.K. (1993) Plant Physiol. 103, 615–620.
[36] Knorpp, C., Hugosson, M., Sjoling, S., Eriksson, AC. and Glaser, E. (1994) Plant Mol. Biol. 26, 571–579.
[37] Glaser, E., Eriksson, AC and Sjoling, S. (1994) FEBS Lett. 346, 83–87.
[38] Nguyen, M., Argan, C., Sheffield, W.P., Bell, A.W., Shields, D., Shore, G.C. (1987) J. Cell Biol. 104, 1193–1198.
[39] Schneider, H.C., Berthold, J., Bauer, H.F., Dietmeier, K., Guiard, B., Brunner, M. and Neupert, W. (1994) Nature 371, 768–774.
[40] Zwinisiki, C. and Neupert, W. (1983) J. Biol. Chem. 258, 13340–13346.
[41] Stzul, E.S., Hendrick, J.P., Kraus, J.P., Wall, D., Kalousek, F. and Rosenberg, L.E. (1987) J. Cell Biol. 105, 2631–2639.
[42] Horwich, A.L., Kalousek, F., Frelen, W.A., Pollock, R.A. and Rosenberg, L.E. (1986) Cell 44, 451–459.
[43] Von Heijne, G., Steppuhn, J. and Herrmann, R.G. (1989) Eur. J. Biochem. 180, 535–545.
[44] Braun, H-P., Jansch, L., Kruft, V., Schmitz, U.K. (1994) FEBS Lett. 347, 90–94.
[45] Braun, H-P., Kruft, V. and Schmitz, U.K. (1994) Biochim. Biophys. Acta 1188, 367–372.
[46] Braun, H-P. and Schmitz, U.K. (1995) Biochim. Biophys. Acta 1229, 181–186.
[47] Braun, H-P. and Schmitz, U.K. (1995) Plant Physiol. 107, 1217–1223.
[48] Winning, B.M., Sarah, C.J., Purdue, P.E., Day, C.D. and Leaver, C.J. (1992) Plant J. 2, 763–773.
[49] Purdue, P.E. (1988) Nuclear genes and protein import into maize mitochondria, Ph. D. Thesis, University of Edinburgh.
[50] Mozo, T., Fischer, K., Flugge, U.I. and Schmitz, U.K. (1995) Plant J. 7, 1015–1020.
[51] Niidome, T., Kitada, S., Shimokata, K., Ogishima, T. and Ito, A. (1994) J. Biol. Chem. 269, 24719–24722.