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Import of precursor proteins into *Vicia faba* mitochondria

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The ADP/ATP translocator from *Vicia faba* and β F_1-ATPase from *Neurospora crassa* were imported into plant mitochondria from a wheat germ and a rabbit reticulocyte translation system. Incubation of *Vicia* mitochondria in postribosomal supernatants of the translation reactions resulted in import of the ADP/ATP translocator. Precursor to the β-subunit of the F_1-ATPase from *N. crassa* was imported and processed by the *Vicia* mitochondria. Import into a protease-protected location was abolished by the inclusion of apyrase or valinomycin in the import mixtures. The *Neurospora* β-subunit precursor showed post-maturation processing to a protein with a molecular mass equivalent to that of the plant β-subunit.

Mitochondria; Protein import; (*Vicia*, Wheat germ)

1. INTRODUCTION

The biogenesis of mitochondria involves the import of translation products of nuclear genes [1–4] as the coding capacity of the mitochondrial genome is so limited [5–7]. Mitochondria must therefore possess a segregating system to allow the selection of mitochondrial protein from the myriad of cytoplasmic proteins and from those destined for the other organelles, in particular chloroplasts. Investigations to date on mitochondrial biogenesis in higher plants have been mainly concerned with the mitochondrial genome and its regulation [8]. Little progress has been made on the study of the import of cytosolically synthesised precursors, despite the strides made in this field with other organisms [9–11].

2. MATERIALS AND METHODS

Mitochondria were isolated from 10-day-old dark-grown hypocotyls according to Douce et al. [12]. RNA was extracted from epicotyls as described by Leaver and Harmey [13]. Poly(A^+) was isolated by applying the crude RNA to an oligo-(dT)-cellulose column [14]. Poly(A^+) RNA was translated in a rabbit reticulocyte lysate [15]. Coupled transcription was performed using *Neurospora* β ATPase cloned in pGem3 by the addition of sp6 RNA polymerase and ribonucleotides to the linearised plasmid; translation of the resultant message was carried out in wheat germ [16] and rabbit reticulocyte lysates.

Import studies were carried out as in [9]. Post-import proteolysis was performed by the application of proteinase K to a final concentration of 200 μg/ml. Immunoprecipitation was performed as described [18], SDS-PAGE was carried out as described by Laemmli [17].

β ATPase cloned into the *Sma*I site of pGEM3 was the kind gift of Dr Max Tropschug (Institut für Physiologische Chemie, Universität München). Antibodies were raised in rabbits and immunoglobulin fractions prepared as in [19].

3. RESULTS

When mitochondria were incubated in the postribosomal supernatant of rabbit reticulocyte lysates programmed with total *Vicia* poly(A^+) RNA, very low levels of import could be demonstrated using the criteria of acquired insensitivity to external proteases. The amount of *Vicia* ADP/ATP translocator which could be immunoprecipitated from the mitochondria and from the total lysate was quite low, suggesting that the amount of message for the protein in question was quite small. The translation products show the presence of relatively high amounts of oligomers

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of the ADP/ATP translocator as has been described for the precursor form of the protein [1].

Fig.1 shows the results of an import experiment carried out on translated poly(A) RNA from *Vicia* hypocotyls followed by immunoprecipitation of the imported ADP/ATP translocator protein. The latter is one of the most abundant of the mitochondrial proteins [17], yet the amount of protease-protected protein was quite low and required long exposure of the autoradiographs to produce the bands shown. Similar experiments were carried out on the import of β F_1-ATPase using antibodies raised against *Neurospora* β ATPase but the levels of import were so low as to be insignificant. (It had been shown previously that the antibody to *Neurospora* β F_1-ATPase recognised the F_1 β from *Vicia*.)

As an enriched source of message for mitochondrial proteins was not available, a viable alternative was provided by using direct transcripts from cDNA for the β-subunit of the F_1-ATPase cloned into pGEM3 which produced a source of message for *Neurospora* β F_1-ATPase. The results of incubating *Vicia* mitochondria in a supernatant from a translation mixture programmed with cDNA transcripts for *N. crassa* β-subunit of the F_1-ATPase are shown in fig.2a. The main translation product was a polypeptide of 58 kDa (precur-

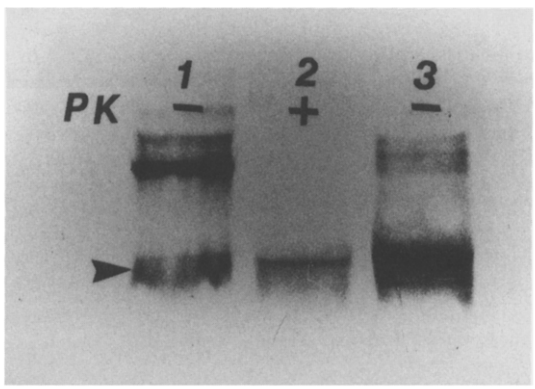

Fig.1. Import of ADP/ATP translocator from a rabbit reticulocyte translation reaction programmed with *Vicia faba* poly(A) RNA. Mitochondria were incubated in the translation mixture diluted with import buffer at 25°C for 30 min. The import reaction mixture was halved, and to one aliquot proteinase K (200 μg/ml) was added, the other being kept on ice. Mitochondria were reisolated, washed, the ADP/ATP translocator immunoprecipitated, subjected to SDS-PAGE and fluorographed. ADP/ATP translocator immunoprecipitated from (lanes): 1, total translation reaction mixture, showing oligomeric and monomeric (32 kDa) forms; 2, post-import mitochondria treated with proteinase K (200 μg/ml). Imported protein only shows the monomeric form (32 kDa); 3, post-import mitochondria minus proteinase K. In the absence of proteinase K the oligomeric forms are still evident, due to association with mitochondrial membranes, as well as imported protein.

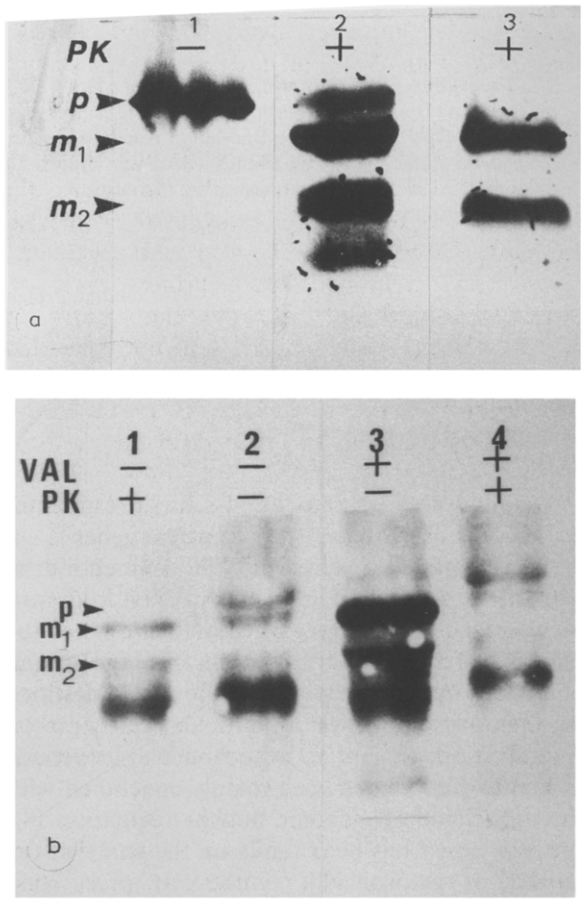

Fig.2. (a) Import of β-subunit of *Neurospora crassa* F_1-ATPase. Lanes: 1, β-subunit synthesised in an in vitro transcription/translation (rabbit reticulocyte) system and separated by SDS-PAGE showing P (58 kDa); 2, import reaction, of post-ribosomal supernatant diluted with 2 × import buffer and *Vicia* mitochondria, treated with proteinase K (200 μg/ml) subjected to SDS-PAGE and fluorography showing P, M1, M2; 3, post-import mitochondria treated with proteinase K (200 μg/ml) subjected to immunoprecipitation with antibody to *Neurospora* β-subunit showing M1, M2. (b) Import of *Neurospora crassa* β-subunit of F_1-ATPase from in vitro transcription/translation (rabbit reticulocyte) reaction into *Vicia* mitochondria in the presence of valinomycin/K^+. Import reaction as for fig.1 with valinomycin (10 μg/ml) as indicated.

sor P). When the labelled mitochondrial proteins which acquired protease insensitivity were analysed three bands were observed. The larger of the bands had an apparent molecular mass of 58 kDa and corresponded to a low amount of precursor (P) which had been imported but not processed. The next band had an apparent molecular mass of 56 kDa (mature M1), which is the value reported for the mature β-subunit of *Neurospora* [18]. This protein is accompanied by a band of 52 kDa (mature M2) which is equivalent to the size of the mature *Vicia* F_1-ATPase β-subunit [23]. When the import products were immunoprecipitated with *Neurospora* F_1 β antibody, both M1 and M2 were precipitated. It would appear therefore that the 52 kDa protein is a specific breakdown product of the mature β polypeptide as it appears to be imported and sequestered into a protease-resistant location but does not appear in the translation mixture alone (fig.2A, lane 1). A lower band with a molecular mass of 50 kDa also appears in the import products, however, it does not appear in the translation reactions with the wheat germ lysate nor is it antigenic to the F_1 β antibody. Addition of valinomycin and K^+ to the import medium abolished the protease insensitivity of both the mature β and the 52 kDa band (fig.2b).

It is well documented that the rabbit reticulocyte lysate contains factors which are necessary for protein import into both *Neurospora* and yeast mitochondria [19]. In view of the phylogenetic gap between *Vicia* and these organisms, we wished to determine whether precursor proteins synthesised in a wheat germ system could also be imported. The experience of others in this field has been that the wheat germ system did not support import (Neupert, personal communication). We translated the transcripts from linearised pGEM3 containing a cDNA for the *Neurospora* β-subunit in a wheat germ lysate and incubated *Vicia* mitochondria in import buffer in the postribosomal supernatant of the translation reaction. The *Neurospora* F_1 β-subunit was imported into a protease-protected location. Preincubation of the reaction mixture with apyrase abolished the protease insensitivity indicating the necessity of ATP for import (fig.3). The lower molecular mass derivative observed when import was performed in rabbit reticulocyte lysate was also present and protease-protected, no evidence of any prominent

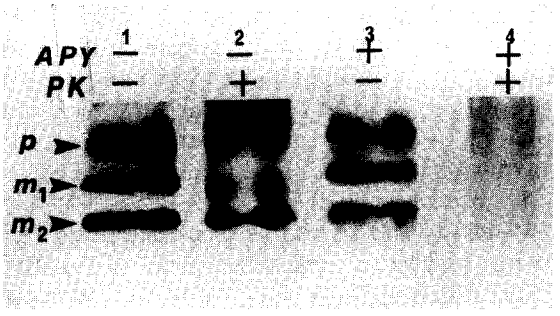

Fig.3. Import of β-subunit of F_1-ATPase from in vitro transcription/translation (wheat germ) reaction into *Vicia* mitochondria in the presence of potato apyrase. Proteinase K and apyrase (0.25 U/ml) were added as indicated.

band other than the β precursor being evident in the translation products of the pGEM transcripts (fig.2a). Addition of reticulocyte lysate to the wheat germ system had no stimulatory effects on import.

4. DISCUSSION

The present results show that import of proteins into plant mitochondria takes place from both rabbit reticulocyte and wheat germ translation reactions. These results are at variance with those of many studying the import process in yeast, *Neurospora* and mammalian mitochondria. In these systems the rabbit reticulocyte lysate has been the translation system of choice [9,10]. The import of protein into yeast mitochondria was dependent on a proteinaceous factor in the lysate [20]. The possibility arises that a closer affinity exists between plant mitochondria and plant translation products. Such an explanation could have validity in respect of the import of the ADP/ATP translocator but hardly in the case of the β-subunit which was translated from message transcribed from cDNA to a *Neurospora* F_1 β clone. The factor present in the reticulocyte lysate would appear to relate more to the maintenance of import competence of the precursor proteins. Import of the F_1 β shares the requirement for membrane potential [19] and ATP [21] described for import by mitochondria in general [1–4].

Boutry et al. [23] have shown that the β-subunit of the F_1-ATPase of *Nicotiana plumbaginifolia* was imported from a coupled transcrip-

tion/translation system [11] and processed to a mature-sized protein with an apparent molecular mass of 52 kDa. The mature F_1 β-subunit of *Vicia* has a molecular mass of 51 kDa [23]. The *Nicotiana* F_1 β precursor has a molecular mass of approx. 60 kDa [11]; the size of the *Vicia* precursor has not been described. Our studies using Northern blot analysis and hybrid release translation (not shown) suggest a molecular mass of approx. 56 kDa. The 52 kDa protein (M2) observed in our import studies has a molecular mass equivalent to that of the mature *Vicia* subunit. The proteolytic processing of many mitochondrial proteins involves a two-step reaction [22]. From the large amount of data on processing of mitochondrial proteins from various sources [24], no clear evidence of sequence homology around the cleavage sites exists. Although presequences are predominantly basic, even presequences containing a high proportion of acidic residues will be cleaved [25]. The matrix-located protease therefore would appear to have the ability to cleave at a wide variety of cleavage sites. The double cleavage event seen in our import studies may reflect this processing ability while structural instability of the *Neurospora* F_1 β-subunit in *Vicia* mitochondria may contribute to this second processing event. The mature protein thus produced may be more compatible on size considerations. In this instance, it is the mature protein which is the substrate for the second proteolytic step and may reflect the result of either a variation in the cleavage specificity of the matrix protease or the instability of non-assembled precursors.

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