Temporal regulation of in vitro import of precursor proteins into tobacco mitochondria

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Received 1 August 1997

Abstract Protein import into isolated tobacco mitochondria was investigated using mitochondria from leaves harvested at different times of the day and night. Efficient import was only detected with mitochondria isolated from leaves harvested during the dark period of the growth cycle, only low levels of import were detected from leaves harvested during the light period. However, this temporal difference seen in import did not appear to be circadian in nature. This implies that the protein import process in mitochondria isolated from leaves is not constitutive. This has important implications for targeting specificity studies performed in transgenic plants, as unless the plants are tested at the time when import is occurring, the true in vivo targeting abilities of chimeric constructs will not be measured.

Key words: Plant mitochondrial import; Regulation; Diurnal rhythm

1. Introduction

The targeting and import of precursor proteins to mitochondria is an intensively studied process. Several components of the import apparatus have been identified and the role they play in the import process dissected with a combination of biochemical and genetic procedures [1–3]. Studies on the plant mitochondrial process have also elucidated several components involved in the import process despite the fact that such studies began 10 years after those in fungal and animal systems [4].

Studies on the import process in a variety of species from different phylogenetic groups indicate that there are general differences in some aspects of the import process. In plants the membrane location of the general mitochondrial processing peptidase (MPP) and the specificity of targeting to mitochondria clearly differ from the situation in yeast [5–8]. Specific differences also exist between different organisms with respect to homologous precursors, examples include the Rieske FeS protein and the adenine nucleotide translocator. The Rieske FeS protein of the cytochrome bc_1 complex in fungi undergoes two-step processing via MPP and the mitochondrial intermediate peptidase (MIP) [9]; single step processing via MPP in bovine with the removed presequence forming part of the cytochrome bc_1 complex [10]; and in plants it is processed in a single step but the presequence does not form part of the bc_1 complex [5]. The adenine nucleotide translocator has a cleavable presequence in plants but not in animals [11,12]. The conclusion from such studies is that to get a general understanding of mitochondrial import it is necessary to understand the process from a variety of species with a range of precursors.

In plants import systems have been developed for a variety of species, specifically maize, potato, spinach and a number of legumes [4,13]. Despite extensive efforts from various groups efficient import of precursor proteins into tobacco mitochondria cannot be achieved ([14–16], Dessi and Whelan, unpublished data). This is all the more surprising as respiratory competent, intact mitochondria capable of in organello protein synthesis can be readily isolated from tobacco leaves [17]. It is desirable to develop an in vitro import system for tobacco mitochondria for two reasons: firstly, targeting specificity studies are carried out in transgenic tobacco and it would be of interest to investigate the basis of this specificity with in vitro studies [18,19]; secondly, with the identification of components of the import apparatus it would be desirable to investigate their role using antisense technology. The value of such studies would be greatly increased if they were complemented with in vitro import assays to see what stage of import was limiting.

In this report we demonstrate that efficient import of precursors into tobacco mitochondria can be achieved. However, import is clearly dependent on the time of tissue harvesting in the daily cycle of light and dark. As well as allowing in vitro import studies into tobacco mitochondria, it reveals a new level of regulation of protein import into plant mitochondria. The implications for such regulation are discussed.

2. Materials and methods

Tobacco plants (Wisconsin 38) were grown in cabinets at a constant 28°C under 65 mmol/m^2/s light intensity with white fluorescent tubes (referred to as white light) or under 32 mmol/m^2/s light intensity with plant growth fluorescent tubes (referred to as growth lights) with a 16 h light and 8 h dark period. Leaves were harvested when they had reached a length of 2 or 6 cm (8 and 20 weeks respectively after germination). Leaves were harvested at the following time points in the photoperiod: after 1, 4, 8, 12 and 16 h of illumination (referred to as L1, L4, L8, L12, and L16 in the text). Leaves were harvested at the following times following the onset of darkness: 1, 4, 6, and 8 h (referred to as D1, D4, D6, and D8 in the text). Mitochondria were isolated from the leaves immediately after harvesting as described above using the method of Day et al. [20]. Import experiments were carried out as previously described [21], using the precursors for the alternative oxidase and the F_Ad subunit of the ATP synthase complex from soybean [22,23]. To ensure no variation in translation lysate batch a large translation mixture was set up to produce sufficient precursor protein for all experiments in a series, i.e. D1, D4, D6, D8, L1, L4, L8, L12 and L16 for both growth conditions. The translation lysate was added to the import mixture to a final volume of 5% (v/v) to ensure a constant amount of various factors that are necessary to support import. Quantitation of the amount of import was carried out using the phosphorimager software (MacBas v2.0 Fuji Photofilm) on raw image scans, obtained by exposing the dried SDS-PAGE gel for 12 h to a Fuji BasTR2040s plate according to manufacturers

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PII S0014-5793(97)01116-2

instructions. To ensure that the signals were in the linear range we exposed various dilutions of both precursors in a similar fashion prior to experiments to encompass the range of signals obtained with the experimental samples and all signals fell within the linear range. The images shown (Fig. 1) represent the unmodified images of the amount of import; however, there will be some variation in the amount of mitochondrial protein in each import reaction. Therefore these results are qualitative in the sense they are not normalised to a standard amount of protein, although a general trend is still evident. The amount of mitochondrial protein in each import reaction was calculated by the Lowry method [24]. Fig. 2 represents the degree of import was expressed as pixel density (pixels per mm^2) per 100 μg of total protein.

In addition to calculating the amount of import on a mitochondrial protein basis, we performed Western blots against three mitochondrial inner membrane proteins, using the same mitochondrial preparations (growth lights) for the imports which are shown in Fig. 1 to investigate if the membrane composition was changing at the different times. 30 μg of total mitochondrial proteins were separated by SDS-PAGE and blotted onto Hybond-C (Amersham, Sydney) and probed with antibodies to the alternative oxidase, cytochrome bc_1 complex and to subunit II of cytochrome *c* oxidase (Molecular Probes, Eugene, OR). Positive bands were visualised using a chemiluminescence detection protocol (Boehringer, Sydney). The signals obtained were quantified by scanning with a U-max scanner and densitometry carried out using ImageQuant 1.11 (Molecular Dynamics). The most intense band for each antibody was expressed as 100%, other values are relative. Again various exposures were used to ensure that bands measured were in the linear range for quantitation.

To check for the presence of a conserved rhythm in the fluctuation of import levels two further experiments were performed. Plants with leaves grown to 6 cm with a 16 h photoperiod from sowing were subjected to either constant illumination or constant darkness for a period of 4 days. Imports were done on mitochondria prepared from leaves harvested at time points equivalent to D4 and L4 on each of the first, second and fourth day.

3. Results

Initially we carried out import experiments on tobacco leaves of varying sizes, ranging from 2 to 12 cm from tip to petiole. Tissue was harvested after 4 h of light exposure (L4). These studies were prompted by some evidence suggesting that in monocot plants mitochondrial biogenesis may be more active in the younger area of leaves rather than the older areas [25,26]. However, only low levels of import were detected, 10% or less than we routinely detect in soybean, spinach or rat liver mitochondria [4]. We then chose two leaf sizes to carry out import studies at different time points over 24 h: 2 and 6 cm size leaves were chosen. 2 cm leaves were chosen as they represented young tissue whereas 6 cm represented mature leaves and sufficient mitochondria could be isolated from a single plant to carry out several import assays.

The import studies carried out at various time points clearly show that isolated tobacco mitochondria can efficiently import precursor proteins. This can be seen in Fig. 1 and is represented graphically in Fig. 2 for both leaf sizes and precursors. A mature protease protected product was evident for both proteins when the mitochondria were isolated during the dark period, in contrast little or no protected precursor or mature forms were evident with mitochondria isolated during the light period (Fig. 1A,B, lane 3 in all panels).

To characterise this apparent difference in import we repeated the assay at least three times for each leaf size at each time point with each precursor, the results are shown in Fig. 2 for an individual experiment. In Fig. 2A,B it can be clearly seen that the alternative oxidase precursor was imported far more efficiently in the dark period than the light period. The smallest difference seen was three-fold, between D6 and L12 for the alternative oxidase with plants grown under growth light (Fig. 2A). In general the difference seen was five-fold (Fig. 2B) and the amount of import detected was equal to that we routinely obtain with soybean or rat liver mitochondrial imports.

In the case of the F_Ad precursor a similar pattern was

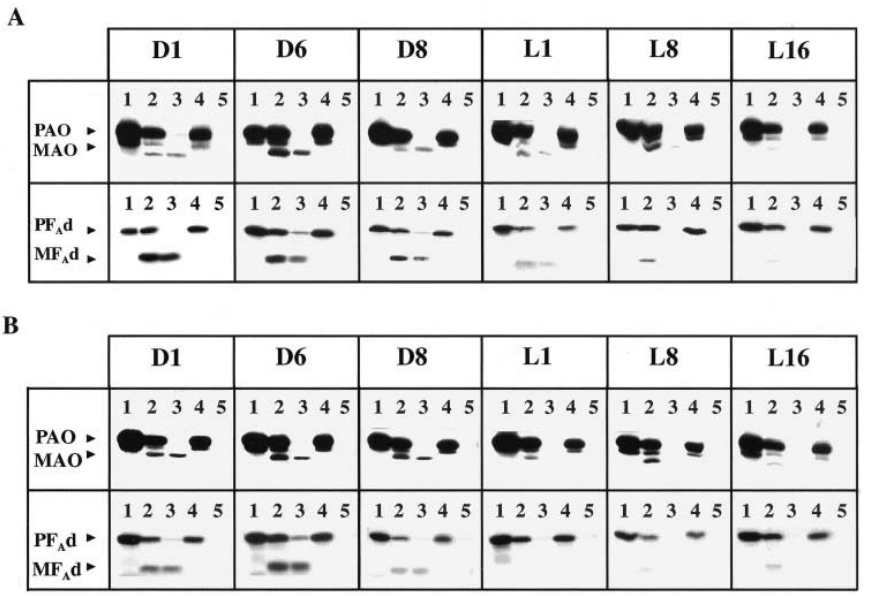

Fig. 1. Import of the alternative oxidase and F_Ad precursors into mitochondria isolated from 6 cm tobacco leaves grown under (A) growth lights and (B) white lights. The top of the panel indicates the time the tissue was harvested for mitochondrial isolation. Lane 1, precursor protein alone, this represents 20% added to the import reactions. Lane 2, precursor protein incubated with mitochondria under conditions that support import. Lane 3, as lane 2 with PK added. Lane 4, as lane 2, with valinomycin added to 1 μM. Lane 5, as lane 4 with PK added. PAO = precursor form of the alternative oxidase, MAO = mature form of the alternative oxidase, PF_Ad = precursor form of the F_Ad subunit and MF_Ad = mature form of the F_Ad subunit.

obtained. However, mitochondria from 2 cm leaves did not import this precursor (data not shown). With 6 cm leaves the import levels detected with this precursor were similar to that seen with the alternative oxidase, with much higher levels of import clearly detected in the dark period (Fig. 2C).

Two aspects of this differential import are worth noting. Firstly, the failure to import was not due to an inability of the mitochondria isolated in the light period to bind either precursor protein. It can be clearly seen in Fig. 1 that lane 2 for the samples harvested in the light display bound precursor, it must be remembered that any apparent variation in binding in Fig. 1 is due to the fact different amounts of protein may be present in the different panels. Binding of precursor based on a protein basis could not account for the low levels of import seen with the light samples (data not shown). Secondly, the failure to import was not due to an inability of the presequence to cross the mitochondrial membranes. Examination of Fig. 1 shows that a mature product for the alternative oxidase is evident in lane 2 of the light time points. We measured the amount of mature product generated for the alternative oxidase under both light conditions and the results are shown in Fig. 3. In contrast to the PK protected imported alternative oxidase generated during the dark period, generation of the mature product in the light was almost as efficient, but this product was evidently not translocated into a protease insensitive location (compare Fig. 2BFig. 3). This point further confirms that the low levels of import seen in the light are not a result of an inability to bind the precursor protein. The processed form of the alternative oxidase generated by mitochondria isolated from the light time points was not due to lysed mitochondria in the import mixture as (i) no such processed products were detected in the valinomycin controls