RESEARCH COMMUNICATION

Rapid degradation of the presequence of the $F_1\beta$ precursor of the ATP synthase inside mitochondria

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We have investigated the fate of the presequence of an over-expressed protein derived from the precursor of the $F_1\beta$ subunit of ATP synthase after import and processing in mitochondria. Our studies revealed a rapid degradation of the presequence inside mitochondria catalysed by matrix-located protease(s). In contrast, the mature portion of the precursor was not degraded.

This is the first experimental evidence of the rapid degradation of a mitochondrial presequence *in organello* after *in vitro* import and processing.

Key words: protein import, proteolysis, signal peptide.

INTRODUCTION

Most mitochondrial proteins are nuclear encoded, synthesized on cytosolic ribosomes as precursor proteins and imported into mitochondria. The great majority of mitochondrial precursors carry targeting information as an N-terminal extension called a signal peptide or presequence [1]. Most of the presequences are proteolytically removed by the general mitochondrial processing peptidase (MPP) [2], after import into mitochondria, to generate the mature functional protein. The presequences are required at various steps along the mitochondrial import pathway, such as interaction with cytosolic and mitochondrial molecular chaperones, binding to the mitochondrial outer-membrane receptors and translocation through the protein translocases of both the outer- and inner-membranes. Finally, the presequence is recognized and cleaved off by MPP.

The length of most mitochondrial presequences is on average 30–40 amino acid residues, but has been shown to vary in the range of 8–123 residues [3,4]. Sequence analysis shows that, despite the lack of obvious sequence identity, the presequences display some common features. They are generally rich in basic and hydroxylated amino acids and have the capacity to form amphiphilic α -helices [5]. In fact, using NMR techniques, synthetic peptides derived from mitochondrial signal peptides were shown to adopt α -helical structures in the presence of micelles [6]. Recently solved, the first NMR structure of the mitochondrial Tom20 receptor (where Tom corresponds to ‘translocase of the outer membrane’) in complex with the presequence peptide of aldehyde dehydrogenase showed that the bound peptide forms an amphiphilic helical structure located in a groove of the receptor [7]. N- and C-termini of the presequences of homologous proteins are generally more conserved compared with middle regions [8]. By analysis of partial presequence deletions and mutations, as well as studies with chimaeric constructs, it has been shown that the N-terminal portion of signal peptides contains information necessary for recognition and import of precursor proteins into mitochondria [9], whereas the C-terminal portion is important for processing by MPP [10]. The import and

processing domains may overlap, especially in short presequences.

Mitochondrial presequences have the ability to disrupt natural and artificial phospholipid bilayers [11]. Addition of presequences to mitochondria results in uncoupling of respiration and a decrease in membrane potential [12]. Furthermore, micromolar concentrations of the $F_1\beta$ presequence peptide have been shown to be toxic to Gram-negative bacteria [12]. Accumulation of presequences inside mitochondria during active protein synthesis and import would cause disruption of the mitochondrial membranes, impaired oxidative phosphorylation and other mitochondrial dysfunctions. Therefore there is a requirement for an effective system to metabolize or remove mitochondrial presequences. The destiny of presequences following cleavage is unknown. Subunit 9 of the mammalian cytochrome bc_1 complex is the only known example where integration of the presequence peptide as a functional subunit of an oligomeric protein complex occurs [13]. There are a few reports concerning mitochondrial import of chemically synthesized short peptides corresponding to either a part of the presequence or the presequence and part of the mature protein [14,15]. The data suggest that degradation of these synthetic peptides occurs inside mitochondria.

We developed a novel procedure for the preparation of chemical quantities of an import-competent mitochondrial precursor [16]. We isolated a soluble import-competent 15 kDa N-terminal fragment of the overexpressed *Nicotiana plumbaginifolia* $F_1\beta$ precursor of the ATP synthase ($N_{15}pF_1\beta$), containing the presequence and the N-terminal portion of the mature protein. Our results showed that $N_{15}pF_1\beta$ retained all of the information necessary for import into mitochondria and processing by MPP, and that $N_{15}pF_1\beta$ can be used for structural and functional studies of the mitochondrial protein import system. The developed procedure has a general value and can be used for the production of chemical quantities of any mitochondrial import substrate and presequence peptide.

In the present study we have investigated the fate of both the presequence and the mature form of $N_{15}pF_1\beta$ upon import into potato tuber mitochondria. We found that the presequence was

Abbreviations used: MPB, 3-(N-maleimidopropionyl)biocytin; MPP, mitochondrial processing peptidase; NEM, N-ethylmaleimide; $N_{15}pF_1\beta$, 15 kDa N-terminal fragment of the overexpressed *Nicotiana plumbaginifolia* $F_1\beta$ precursor of the ATP synthase; SSB, sulphosuccinimidyl biotin.

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rapidly degraded, whereas the mature form was stable inside mitochondria. The degradation activity that rapidly degrades the presequence was found in the matrix, and was ATP-dependent and sensitive to a broad spectrum of protease inhibitors. Our data suggest that degradation is a general way for mitochondria to neutralize potentially harmful presequences.

MATERIALS AND METHODS

Preparation of N_{15} pF $_1\beta$ and biotin- N_{15} pF $_1\beta$

The precursor of the F $_1\beta$ subunit of the mitochondrial ATP synthase, pF $_1\beta$, from *N. plumbaginifolia* was overexpressed in *Escherichia coli* BL21(DE3) [16]. During overexpression of the precursor, pF $_1\beta$ was collected in inclusion bodies that were purified according to Pavlov et al. [16]. The overexpressed protein was cleaved with CNBr and purified using ion-exchange chromatography to obtain an import-competent precursor, N_{15} pF $_1\beta$. N_{15} pF $_1\beta$ was then modified with 3-(*N*-maleimido-propionyl)biocytin (MPB), yielding (Cys)biotin- N_{15} pF $_1\beta$, as described previously [16]. Biotinylation of N_{15} pF $_1\beta$ with sulphosuccinimidyl biotin (SSB), yielding (Lys)biotin- N_{15} pF $_1\beta$, was performed in 10 mM Mops/KOH (pH 7.5) at 25 °C for 1 h. Biotinylated N_{15} pF $_1\beta$ was separated from unreacted MPB/SSB by gel-filtration on PD-10 columns.

Mitochondrial *in vitro* import and processing

Potato tuber mitochondria were isolated and import experiments were performed as described previously [16], using 200 μ g of mitochondrial protein. Import of N_{15} pF $_1\beta$ or biotin- N_{15} pF $_1\beta$ was performed with 0.01 nM precursor protein. Valinomycin (1 μ M) was used to stop the import reaction. To digest proteins outside of the mitochondria, samples were treated with thermolysin (100 μ g/ml) for 30 min at 4 °C. The proteolytic reaction was inhibited by the addition of 5 mM EDTA. Mitochondria were re-isolated and subjected to SDS/PAGE. The MPP/cytochrome *bc*₁ complex from *Spinacia oleracea* was purified, and processing experiments were performed as described previously [16]. N_{15} pF $_1\beta$ or biotin- N_{15} pF $_1\beta$ (0.01 nM) was incubated with 0.25 μ g of MPP/cytochrome *bc*₁ complex for 1 h at 30 °C. The processing reaction was stopped by the addition of 5 mM 1,10-*o*-phenanthroline.

Degradation of processing products of N_{15} pF $_1\beta$ or (Cys)biotin- N_{15} pF $_1\beta$

Mitochondria were solubilized in 10 mM Hepes and 1 % (v/v) Triton X-100, pH 8.0. N_{15} pF $_1\beta$ and (Cys)biotin- N_{15} pF $_1\beta$ were processed with isolated MPP as described above. Degradation assays contained processing products of N_{15} pF $_1\beta$ or (Cys)biotin-

N_{15} pF $_1\beta$ (0.01 nM each) and solubilized mitochondria (33 μ g), in a buffer containing 10 mM Hepes, 1 % Triton X-100 and 1 mM MnCl₂, pH 8.0. For kinetic studies samples were incubated at 15 °C or 25 °C for 10, 20 or 30 min. The effect of inhibitors on the proteolytic activity was investigated using solubilized mitochondria preincubated (10 min at 4 °C) with 1 mM PMSF, 1 mM *N*-ethylmaleimide (NEM), 1 μ M epoxysuccinyl-leucyl-amido-(4-guanidino)butane, 150 μ M bestatin, 20 mM 1,10-*o*-phenanthroline or 100 units/ml apyrase. Potato tuber mitochondrial membrane and matrix fractions were obtained by sonication for 3 $\times$ 15 s on ice, followed by ultracentrifugation at 200 000 *g* for 30 min. For inhibitory studies, processing products were incubated with mitochondrial lysate, membrane or matrix for 30 min at 15 °C.

SDS/PAGE and Western blotting

Samples were analysed by SDS/PAGE using 12–20 % polyacrylamide gels, in the presence of 4 M urea. Immunological cross-reactivity was analysed by Western blotting using Hybond nitrocellulose membranes that were either immunodecorated with antibodies raised against the C-terminal part of the presequence (residues 38–54) of pF $_1\beta$, followed by detection with horseradish peroxidase-labelled secondary antibodies and enhanced chemiluminescence (Amersham), or directly decorated using the avidin–peroxidase conjugate. The X-ray films were scanned with a Molecular Dynamics Personal Densitometer. Degradation was estimated as the ratio between the presequence/mature form and the sum of the presequence/mature and precursor forms.

RESULTS

The overexpression of mitochondrial precursor proteins in *E. coli* is a useful technique to obtain chemical quantities of precursors to study the mitochondrial protein import system. We prepared chemical amounts of N_{15} pF $_1\beta$ [16]. This fragment of 136 amino acids consisted of a 54 amino acid long presequence and the first 82 amino acids from the N-terminal part of the mature portion of the F $_1\beta$ subunit (Figure 1). The mature part contained one cysteine residue at position 93 that was the target for biotinylation with MPB. Lysine residues at positions 32, 44, 70, 78 and 91 were a possible target for biotinylation with SSB. By using antibodies raised against the C-terminal part of the presequence, in combination with an avidin–peroxidase detection system, we could trace both the presequence and the mature F $_1\beta$ portion, respectively, during mitochondrial protein import. Figure 2 shows both *in vitro* processing and import of N_{15} pF $_1\beta$ and biotin- N_{15} pF $_1\beta$. Processing of N_{15} pF $_1\beta$ and (Cys)biotin- N_{15} pF $_1\beta$ was performed by incubation of the precursor with isolated

Figure 1 Schematic representation of pF $_1\beta$

pF $_1\beta$ has a 54 amino acid long presequence. CNBr cleavage at the N-terminus of pF $_1\beta$ occurred at methionine residues at positions 136 and 142. Biotinylation of N_{15} pF $_1\beta$ with MPB occurred at cysteine residue 93. Lysine residues at positions 32, 44, 70, 78 and 91 are possible targets for biotinylation with SSB.

Figure 2 *In vitro* processing and import of biotin-N₁₅pF₁β and N₁₅pF₁β

Western blot analysis using avidin-peroxidase (**A**, **B** and **E**) and antibodies directed against the C-terminal part of the presequence of pF₁β (**C** and **D**). (**A**) Processing of (Cys)biotin-N₁₅pF₁β with isolated spinach MPP/cytochrome *bc*₁ complex as described in the Materials and methods section. (**B**) Import of (Cys)biotin-N₁₅pF₁β into isolated potato tuber mitochondria as described in the Materials and methods section. (**C** and **D**) as in **A** and **B**, respectively, but with N₁₅pF₁β. (**E**) Processing and import of (Lys)biotin-N₁₅pF₁β, as in **A** and **B**.

spinach MPP. The processing reaction generated fragments of 8.5 kDa and 5.5 kDa, corresponding to the mature part (Figure 2A) and the presequence (Figure 2C) of N₁₅pF₁β respectively. The processing reaction was completely inhibited by 5 mM 1,10-*o*-phenanthroline, a specific inhibitor of MPP. Incubation of (Cys)biotin-N₁₅pF₁β with potato tuber mitochondria resulted in import and processing of the precursor (Figure 2B). Both the precursor and the mature form were protected inside mitochondria after incubation of mitochondria with an externally added protease, thermolysin. The addition of valinomycin abolished import and no protease resistant fragments could be detected. In contrast, the presequence was not detected under import conditions either before or after treatment of mitochondria with thermolysin, or in the presence of valinomycin (Figure 2D).

These results could be interpreted to indicate a rapid degradation of the presequence inside mitochondria. To confirm degradation of the presequence we performed labelling of N₁₅pF₁β with SSB at lysine residues (positions 32 and 44 on the presequence and positions 70, 78 and 91 on the mature portion), yielding (Lys)biotin-N₁₅pF₁β. The results of *in vitro* processing and mitochondrial import of (Lys)biotin-N₁₅pF₁β are presented in Figure 2E. Processing of (Lys)biotin-N₁₅pF₁β produced fragments of 9 kDa and 6 kDa corresponding to both the biotinylated mature part of the protein and the presequence (Figure 2E, lane 2). Import of (Lys)biotin-N₁₅pF₁β revealed the presence of only the mature form inside mitochondria, showing that the presequence was degraded (Figure 2E, lanes 3 and 4). Due to an incomplete labelling of all available lysines and diffusion of the modified processing products, we did not use (Lys)biotin-N₁₅pF₁β for the quantification of presequence degradation.

To investigate the susceptibility of the presequence and the mature form to proteolytic breakdown, N₁₅pF₁β and (Cys)biotin-N₁₅pF₁β were processed with the MPP/cytochrome *bc*₁ complex and added to solubilized mitochondria in the presence of Triton X-100. We studied the effect of different temperatures and incubation times on proteolysis of the presequence and of the mature form (Figure 3). No degradation of the mature form was detected after 30 min of incubation at 15 °C or 25 °C (Figure 3A). In contrast, the presequence was rapidly degraded (Figure 3B). Almost complete degradation of the presequence was achieved after a 10 min incubation at 25 °C. At 15 °C the degradation was 25 % at 10 min, 55 % at 20 min and 84 % at 30 min. These values give a half-life of 18 min at 15 °C. No proteolytic fragment(s) were observed upon degradation of the presequence and detection with either C-terminal antibodies or by avidin-peroxidase coupled to the biotinylated presequence (Figures 2D, 2E and 3B).

To investigate protease(s) responsible for the degradation of the presequence we investigated the sensitivity of proteolysis to different inhibitors. The proteolytic activity in the mitochondrial lysate was partially sensitive to a variety of protease inhibitors [NEM (55 %), PMSF (28 %) and 1,10-*o*-phenanthroline (65 %)], affecting different classes of proteases such as cysteine-, serine- and metalloproteases.

In order to determine the intramitochondrial location of the proteolytic event we performed fractionation of mitochondria and analysed matrix and membrane fractions for the proteolytic activity (Figure 4). Complete degradation was observed upon incubation of the MPP-processed N₁₅pF₁β (30 min at 15 °C) with the matrix fraction (Figure 4A), whereas almost no degradation was achieved with the membrane fraction (Figure 4B). The matrix-located proteolytic activity was sensitive to the same inhibitors as the mitochondrial lysate, with a stronger inhibitory effect. Furthermore, the matrix-located degradation activity

Figure 3 Time and temperature dependence of the degradation of the presequence and mature part of N₁₅pF₁β with mitochondrial lysate

(A) Western blot analysis using avidin–peroxidase. (B) Western blot analysis using antibodies directed against the C-terminal part of the presequence of pF₁β. (Cys)Biotin-N₁₅pF₁β and N₁₅pF₁β were incubated with mitochondrial lysate as described in the Materials and methods section.

was partially sensitive to apyrase, suggesting that the activity was ATP-dependent.

DISCUSSION

In the present study we have demonstrated a rapid degradation of a mitochondrial presequence inside mitochondria. We have also shown that there is a substantial difference in the efficiency of the presequence degradation compared with degradation of the mature part of the imported precursor protein, N₁₅pF₁β. This is the first experimental evidence for the rapid degradation of a

mitochondrial presequence *in organello* after *in vitro* import and processing of the overexpressed precursor. We obtained similar results using the precursor of *Glycine max* alternative oxidase, with the presequence containing six methionine residues, synthesized *in vitro* and labelled with [³⁵S]methionine (results not shown), indicating that rapid degradation of presequences inside mitochondria is a general phenomenon.

We localized the rapid degradation event to the matrix. Only minor proteolysis was observed in the membrane. To assess the physiological relevance of presequence degradation, the location of the presequence inside mitochondria has to be considered. In yeast and mammals MPP is located entirely within the matrix [17]. Despite the fact that the plant MPP is integrated into the cytochrome *bc*₁ complex of the respiratory chain, the MPP proteolytic subunits protrude into the matrix and expose their catalytic site at a distal location to the membrane, resulting in presequence cleavage in the matrix phase. Amphiphilic, positively charged mitochondrial presequences are soluble. However, they show high binding affinity to biological membranes, making them potentially harmful to the membranes. Rapid degradation of presequences in the matrix directly after MPP cleavage would thus neutralize the toxic effect of presequences on mitochondrial membranes. The matrix-located proteolytic activity showed sensitivity to a broad spectrum of protease inhibitors, suggesting the involvement of several proteases in presequence degradation *in organello*. The degradation of synthetic presequence peptides is differently affected by various protease inhibitors [18,19]. The matrix-located degradation activity was also ATP-dependent. These results taken together might reflect the presence of different pathways for presequence degradation.

Under the experimental conditions used to detect presequence degradation we have not seen any proteolytic fragments. Members of the oligopeptidase family of proteases with the ability to degrade small peptides like dinorphin and neurotensin were identified in mammalian and yeast mitochondria [20,21]. However, the location of these oligopeptidases in the inter-membrane space of mitochondria makes mitochondrial presequences unlikely to be their substrates. The matrix PIM1 serine protease (a homologue of the bacterial Lon protease) and the inner-membrane metalloproteases, Yta10 and Yta12 (members of the AAA-protease family, homologues of the bacterial FtsH protease), have been shown to degrade different matrix and

Figure 4 Degradation of the presequence by matrix or membrane fractions

Western blot analysis using antibodies directed against the C-terminal part of the presequence of pF₁β. (A) N₁₅pF₁β was incubated with matrix fractions as described in the Materials and methods section. (B) N₁₅pF₁β was incubated with membranes as described in the Materials and methods section.

inner-membrane protein complexes in an ATP-dependent manner [22]. In bacteria the soluble ClpP (caseinolytic protease) serine protease degrades abnormal proteins in an ATP-dependent manner. A ClpP homologue has been identified in human mitochondria with unknown function [23]. A partial ATP-dependency and a broad inhibitory sensitivity suggest the involvement of an ATP-dependent complex proteolytic system in the degradation of presequences.

What protease(s) may thus be responsible for the rapid degradation of presequences in the matrix? An interesting observation is that the presequence attached to the mature protein remains stable until cleaved by MPP. One obvious difference between the free and the bound presequence is the presence of a free carboxy group at the C-terminus of the presequence. On the basis of this observation the involvement of carboxypeptidase(s) in the degradation of presequences can be proposed. Structural differences between the presequence attached to the passenger protein and the free form of the presequence, as well as proteases able to discriminate between these forms, might be responsible for different stabilities of cleaved and uncleaved presequences. The presequence, being exposed in aqueous solution, has limited impact on the folding, catalytic activity and stability of the mature protein *in vitro* [24]. On the other hand the passenger protein masks the amphiphilic properties of the presequence [25]. The above reasoning, as well as the lack of proteolytic fragments during degradation, suggests the involvement of exopeptidase(s), possibly carboxypeptidase(s) in presequence degradation or in initiation of the degradation process. This is in contrast to the degradation of chloroplast signal peptides by stromal extracts where smaller subfragments are generated and subsequently degraded [26].

Our data suggest that degradation of cleaved presequences inside mitochondria is a general fate of mitochondrial presequences. This process might contribute to the signalling between the mitochondria and the nucleus via the products of presequence catabolism. Amino acids recovered from the degraded presequence can be utilized for synthesis of mitochondrial proteins or further catabolized via the urea cycle. Arginine is one of the most abundant amino acids found in presequences and is also a precursor for the synthesis of nitric oxide, polyamines and other biologically active molecules. The arginine-converting enzymes like arginase and nitric oxide synthase have been identified in mitochondria [27,28]. This raises the possibility that an excess of arginine from presequence catabolism might be converted into nitric oxide and regulate transcription of nuclear encoded mitochondrial genes, providing cross talk between mitochondria and the nucleus.

This work was supported by grants from the Swedish Natural Science Research Council and from The Swedish Foundation for Strategic Research. We are grateful to Dr Patrick Dessi for comments on the manuscript.

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