Chemical cleavage of the overexpressed mitochondrial $F_1\beta$ precursor with CNBr: a new strategy to construct an import-competent preprotein

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We have isolated a soluble import-competent 15 kDa N-terminal fragment of the overexpressed *Nicotiana plumbaginifolia* $F_1\beta$ precursor of the ATP synthase ($N_{15}pF_1\beta$). The isolation was achieved after chemical cleavage, with CNBr, of the insoluble precursor collected in inclusion bodies, followed by purification of the fragment using ion-exchange chromatography. The purity of the final product was estimated to be more than 99%. $N_{15}pF_1\beta$ contained a presequence of 54 amino acid residues (except for the N-terminal methionine residue) and 82 N-terminal residues of the mature protein. $N_{15}pF_1\beta$ was shown to be imported into isolated potato tuber mitochondria and to be processed by the isolated mitochondrial processing peptidase (MPP) integrated into the cytochrome bc_1 complex of the respiratory chain. Addition of $N_{15}pF_1\beta$ at micromolar concentrations resulted in the inhibition of import of $F_1\beta$ precursor and alternative oxidase precursor, synthesized *in vitro*, into isolated mitochondria as well as the processing of these precursors catalysed by the isolated MPP–bc_1 complex. $N_{15}pF_1\beta$ conjugated via a biotin link to avidin blocked import sites even after the reisolation of mitochondria and inhibited the import of the mitochondrial precursors, indicating that it can be used as a substrate for the generation of a stable translocation intermediate. Our results present a novel procedure for the production of an N-terminal fragment of the $F_1\beta$ precursor that contains all information necessary for mitochondrial targeting and processing and that can be used for structural and functional studies of the mitochondrial protein import system. This procedure has a general value because it can be used for the production of chemical quantities of any mitochondrial import substrate and presequence peptide.

Key words: avidin–biotin technique, cytochrome bc_1 complex, *Escherichia coli* overexpression, plant mitochondria, processing, protein import.

INTRODUCTION

The mitochondrial genome encodes only a small number of its own proteins, whereas the vast majority of the mitochondrial proteins are nuclear encoded, translated on cytosolic polyribosomes and imported into mitochondria. Mitochondrial protein import is a very complex multistep process that requires the co-operative action of molecular chaperones with the components of outer membrane (TOM) and inner membrane (TIM) translocase machinery [1,2]. The existence of two mitochondrial membranes causes an additional level of complexity: preproteins have to be sorted between different mitochondrial subcompartments. A network of the signals and sorting pathways delivering preproteins to different compartments inside mitochondria still has to be determined. Usually, preproteins destined for the mitochondrial matrix and some proteins of the inner membrane and intermembrane space are synthesized with a cleavable N-terminal extension called the presequence, which has the capacity to form a positively charged amphiphilic α-helix [3]. The presequence interacts with cytosolic chaperones and import-stimulating factors as well as with acidic domains of receptors on the surface of outer and inner mitochondrial membranes [4–6]. The presequence was also shown to have an important role in membrane-potential-dependent initiation of translocation of the precursor through the inner mitochondrial membrane [7]. Targeting signals within precursor proteins often reflect specific intramitochondrial import pathways. Indeed, some mitochondrial precursors have an additional sorting signal within the mature portion of the protein; some do not contain a cleavable presequence or do not show any similarity to the predicted positively charged amphiphilic α-helix [8–10]. The folding state and structural elements within the mature portion of the preprotein also seem to have an important role in the import process. For example, import of the yeast $F_1\beta$ precursor ($pF_1\beta$) with an internal deletion [Δ-(94–381)] showed no dependence on externally added ATP that is presumably utilized by cytosolic molecular chaperones, whereas import of the wild-type $pF_1\beta$ required external ATP. The effect of ATP could not be overcome by denaturation with urea [11]. In addition, the N-terminus of the mature $F_1\beta$ from yeast was shown to act as an intramolecular chaperone and to facilitate protein import into mitochondria [12]. Striking differences in the rates of mitochondrial import between reticulocyte lysate and wheat-germ-translated preproteins might be explained in terms of the incorrect folding of the precursors. The few precursors that can be imported into mitochondria from wheat-germ extract were shown either to be permanently unfolded or not to require the addition of external ATP for import [13,14]. Overexpression of mitochondrial precursors in *Escherichia coli* frequently results in the production of preproteins packed in insoluble, inactive inclusion bodies. This might be due to the potential toxicity of mitochondrial presequences for bacteria [15]. Properties of the

Abbreviations used: DHFR, dihydrofolate reductase; MPB, 3-(*N*-maleimidopropionyl)biocytin; MPP, mitochondrial processing peptidase; Hsp70, 70 kDa heat-shock protein; $N_{15}pF_1\beta$, 15 kDa N-terminal $pF_1\beta$ fragment; pAOX, alternative oxidase precursor; $pF_1\beta$, $F_1\beta$ precursor; TIM, translocase of inner membrane; TOM, translocase of outer membrane.

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mitochondrial presequences are similar to those of anti-microbial peptides produced by insects and animals, and presequences were shown to be toxic for Gram-negative bacteria at micromolar concentrations [15]. The yeast $F_1\beta$ preprotein isolated from inclusion bodies was import-incompetent but it could be solubilized in 8 M urea and used in protein import studies [16]. An overexpressed and urea-solubilized precursor [a precursor protein consisting of the cytochrome oxidase subunit IV presequence fused to the entire dihydrofolate reductase (DHFR) protein, in turn fused to avidin] has been reported to be imported into mitochondria only after preincubation with a cytosolic fraction [17].

In the present study we have investigated properties of the overexpressed *Nicotiana plumbaginifolia* $pF_1\beta$ and developed a new strategy for the production of an import-competent precursor protein. We found that full-length $pF_1\beta$ isolated from inclusion bodies remained import-incompetent even after solubilization in 8 M urea and preincubation with rabbit reticulocyte lysate before import. The 15 kDa N-terminal fragment of the $pF_1\beta$ ($N_{15}pF_1\beta$) obtained by cleavage of overexpressed $F_1\beta$ preprotein with CNBr was found to contain sufficient information for mitochondrial targeting on its own. This fragment can be used for mitochondrial protein import and processing studies, especially with an avidin–biotin technique, as a tool for the generation of translocation intermediates.

MATERIALS AND METHODS

Overexpression and purification of $pF_1\beta$

The expression $pF_1\beta$ plasmid was constructed by inserting two-thirds of the cDNA [from the ATG start site (*Nco*I site) to the *Sal*I site (residues 1–513)] of the β-subunit of mitochondrial ATPase from *N. plumbaginifolia* into pET-21d plasmid. The cDNA clone was originally obtained from Boutry and Chua [18]. An overexpression strain BL21(DE3) was transformed with the newly constructed plasmid and grown at 30 °C as described in [19]. Expression of $pF_1\beta$ was achieved by induction with isopropyl β-D-thiogalactoside at a final concentration of 1 mM for 3–4 h at 30 °C. The overexpressed protein was insoluble and present in inclusion bodies. The purification of the inclusion bodies was performed as described in [19], with small modifications. At least 25 mg of inclusion bodies can be purified from 1 litre of bacterial culture.

Preparation of $N_{15}pF_1\beta$

The overexpressed $pF_1\beta$ was precipitated with 50 % $(NH_4)_2SO_4$ and centrifuged at 10000 ***g*** for 10 min. The pellet was solubilized in 70 % formic acid; a 100-fold molar excess of CNBr in relation to the methionine was added. Incubation was performed overnight in the dark at 25 °C. After incubation, the sample was evaporated under vacuum, redissolved in a buffer containing 10 mM Tris/HCl, 1 mM dithiothreitol and 4 M urea (pH adjusted to 8.0 with NaOH) and applied to a CM-Sephadex column equilibrated with 10 mM Tris/HCl/4 M urea (pH 8.0). Elution was performed with 0.5 M NaCl. Fractions after chromatography were analysed by SDS/PAGE and fractions containing $N_{15}pF_1\beta$ were collected and concentrated with a Microsep ultrafiltration membrane.

Potato tuber mitochondria and mitoplasts

These were prepared as described previously [20].

Spinach leaf mitochondria

These were prepared as described previously [21] and the mitochondrial processing peptidase (MPP)–cytochrome bc_1 complex was purified as described in [22].

Membrane potential measurements

These were performed with Rhodamine 123 as described in [23]. The final concentration of mitochondrial protein was 1 mg/ml.

Modification of $N_{15}pF_1\beta$ with 3-(N-maleimidopropionyl)biocytin (MPB) and generation of an avidin–biotin-labelled fragment complex

10 mM Tris/HCl/2 M urea (pH 8.0) buffer (0.5 ml) containing $N_{15}pF_1\beta$ (0.35 mg/ml) was incubated with 1 mM MPB for 30 min at 25 °C followed by inactivation by 10 mM dithiothreitol for 5 min. The solution was diluted 1:10 and $N_{15}pF_1\beta$ was re-isolated with CM-Sephadex chromatography as described above. An avidin–biotin-labelled $N_{15}pF_1\beta$ complex was formed by mixing MPB-labelled $N_{15}pF_1\beta$ and egg-white avidin in the molar ratio 1:4 and incubating for 15 min at 0 °C.

Synthesis of the precursor proteins *in vitro*

A pTZ18U plasmid containing cDNA of the $pF_1\beta$ subunit of ATPase from *N. plumbaginifolia* [18] and a plasmid containing the cDNA clone of soybean alternative oxidase precursor (pAOX) (a gift from J. Whelan) were used for the expression of precursor proteins *in vitro*. Proteins were produced in a coupled transcription-linked translation reticulocyte lysate TNT system (Promega, Madison, WI, U.S.A.) in the presence of ^{35}S-labelled methionine (Amersham, Little Chalfont, Bucks., U.K.) in accordance with the Promega protocol.

Mitochondrial import *in vitro*

Protein import experiments were performed as described previously [20], with 200 μg of mitochondrial protein. To study the effect of $N_{15}pF_1\beta$ on the import of $pF_1\beta$ and pAOX synthesized *in vitro*, mitochondria were preincubated with this fragment for 5 min at 0 °C before the addition of proteins produced in a transcription-linked translation system *in vitro*. Import of $N_{15}pF_1\beta$–MPB was performed with 50 nM of the precursor protein. PMSF (1 mM) was added to the import buffer to prevent the degradation of $N_{15}pF_1\beta$–MPB inside the mitochondria. After completion of the import process, treatment with thermolysin (100 μg/ml for 30 min at 4 °C) was performed to digest proteins outside the mitochondria. Protease activity was inhibited by the addition of 5 mM EDTA. In control experiments, valinomycin was added at 1 μM. In experiments in which a stable translocation intermediate was generated, mitochondria were preincubated for 15 min at 15 °C with the avidin–biotin-labelled $N_{15}pF_1\beta$ complex, sedimented and washed twice with the import buffer, then resuspended in import buffer and subjected to import. All inhibitory studies and experiments with translocation intermediates were performed at least three times.

Processing *in vitro*

Processing *in vitro* catalysed by the isolated MPP–bc_1 was performed as described in [22]. $N_{15}pF_1\beta$ or $N_{15}pF_1\beta$–MPB (25 nM) was incubated with 0.05 nM purified MPP–bc_1 complex for 2 h at 30 °C. *o*-Phenanthroline was added at 5 mM when indicated. For competition studies, increasing amounts of

$N_{15}pF_1\beta$ were preincubated with MPP–bc_1 for 5 min before the addition of radiolabelled precursors synthesized *in vitro*.

SDS/PAGE and Western blotting

Samples were analysed by SDS/PAGE in the presence of 4 M urea [24]. Western blots on nitrocellulose membranes were either immunodecorated with antibodies raised against a peptide (residues 40–54) corresponding to the C-terminal part of the presequence of $pF_1\beta$ and detected with horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminiscence (ECL®; Amersham), or directly decorated by using the avidin–peroxidase conjugate.

RESULTS

Preparation of chemical quantities of $N_{15}pF_1\beta$

The overexpression of mitochondrial preproteins in *E. coli* is a useful technique for obtaining precursors in chemical quantities to study the mitochondrial protein import system. In attempts to obtain a satisfactory overexpression of $pF_1\beta$ we examined several *E. coli* strains. *E. coli* strain BL21(DE3) transformed with plasmid pET-21d for the expression of $pF_1\beta$ produced large amounts of the insoluble protein packed into inclusion bodies (Figure 1). As much as 25–50 mg of inclusion bodies could be isolated from 1 litre of *E. coli* culture. Our attempts to produce soluble $pF_1\beta$ with this strain or other *E. coli* strains were unsuccessful; the $pF_1\beta$ could be solubilized in 8 M urea but it was not suitable for import studies as the protein became aggregated at urea concentrations of less than 2 M. The isolated, overexpressed $pF_1\beta$ did not compete with radiolabelled $pF_1\beta$ and pAOX synthesized *in vitro* for the common import pathway into mitochondria. To optimize import conditions we preincubated the precursor with rabbit reticulocyte lysate, which naturally contains cytosolic chaperones such as 70 kDa heat-shock protein (Hsp70) and import-stimulating factors (reviewed in [25]); however, no stimulation of import could be observed (results not shown). Preparation of a large fragment of $pF_1\beta$ might be a useful method for the production of a soluble protein suitable for studies of mitochondrial import and processing. Chemical cleavage of $pF_1\beta$ with CNBr results in the fragmentation of a polypeptide chain at positions after methionine residues. Figure 2 presents a schematic illustration of the $pF_1\beta$ protein. The first methionine residue from the $pF_1\beta$ N-terminus (excluding the methionine residue at the N-terminus) is positioned at a distance of 136 residues from the $pF_1\beta$ N-terminus. Therefore after cleavage with CNBr we expected a 14–15 kDa $pF_1\beta$ N-terminal fragment containing the presequence (2–54 residues) and 82 residues of the mature $F_1\beta$. The overexpressed $pF_1\beta$ has 10 methionine residues but other CNBr cleavage fragments were predicted to be much smaller and to not exceed 5 kDa. Prediction of the isoelectric point of the N-terminal fragment at the basic region (pI 10) suggested that ion-exchange chromatography could be used for its purification. We used cation-exchange chromatography on a CM-Sephadex G-25 column at pH 8 to bind the N-terminal fragment to the column; most of the other fragments passed through the column. Results presented in Figure 3 show the purification of $N_{15}pF_1\beta$. Chemical cleavage with CNBr resulted in the complete degradation of $pF_1\beta$ (Figure 3B, lanes 1–3); several fragments with molecular masses of 3–20 kDa appeared on SDS/PAGE. At the same time only fragments with a molecular mass of 15 and 15.5 kDa were recognized by antibodies raised against a peptide representing the C-terminal part of the $pF_1\beta$ presequence (residues 40–54) (Figure 3C, lanes 1–3). The 15.5 kDa band seems to represent a larger version of $N_{15}pF_1\beta$ as a product of incomplete CNBr cleavage of $pF_1\beta$ at methionine-142 (Figure 2). This peptide had virtually the same properties as $N_{15}pF_1\beta$ and was usually present in relatively small amounts (less than 20 %) in comparison with $N_{15}pF_1\beta$. Fragments were applied to the CM-Sephadex column. Figure 3(A) presents a protein elution profile with a linear gradient (0–0.5 M) of NaCl. Fractions were collected and analysed by SDS/PAGE. $N_{15}pF_1\beta$ was found without detectable impurities in fractions eluted with

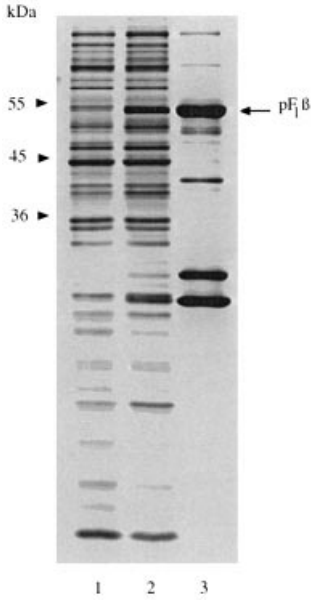

Figure 1 Overexpression and purification of $pF_1\beta$

Plasmid pET-21d, containing the gene of $pF_1\beta$ of ATP synthase from *N. plumbaginifolia*, was transformed into *E. coli* BL21(DE3) cells for overexpression. The expression and purity of $pF_1\beta$ isolated from inclusion bodies were analysed by SDS/PAGE. Lane 1, total cell lysate from uninduced *E. coli*; lane 2, cell lysate 3 h after induction with isopropyl β-D-thiogalactoside; lane 3, purified inclusion bodies, containing 55 kDa $pF_1\beta$, solubilized in 8 M urea.

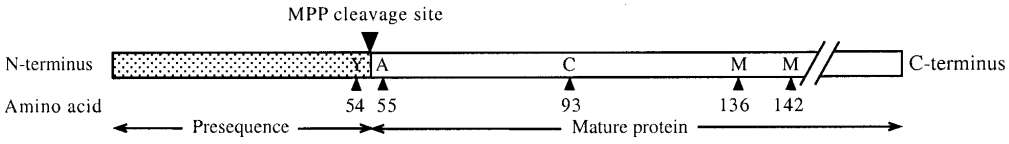

Figure 2 Diagram of $pF_1\beta$

$pF_1\beta$ has an N-terminal presequence of 54 residues. The first two cleavage sites for CNBr in the $pF_1\beta$ are the methionine residues at positions 136 and 142. Biotin-labelling by MPB occurred at Cys-93.

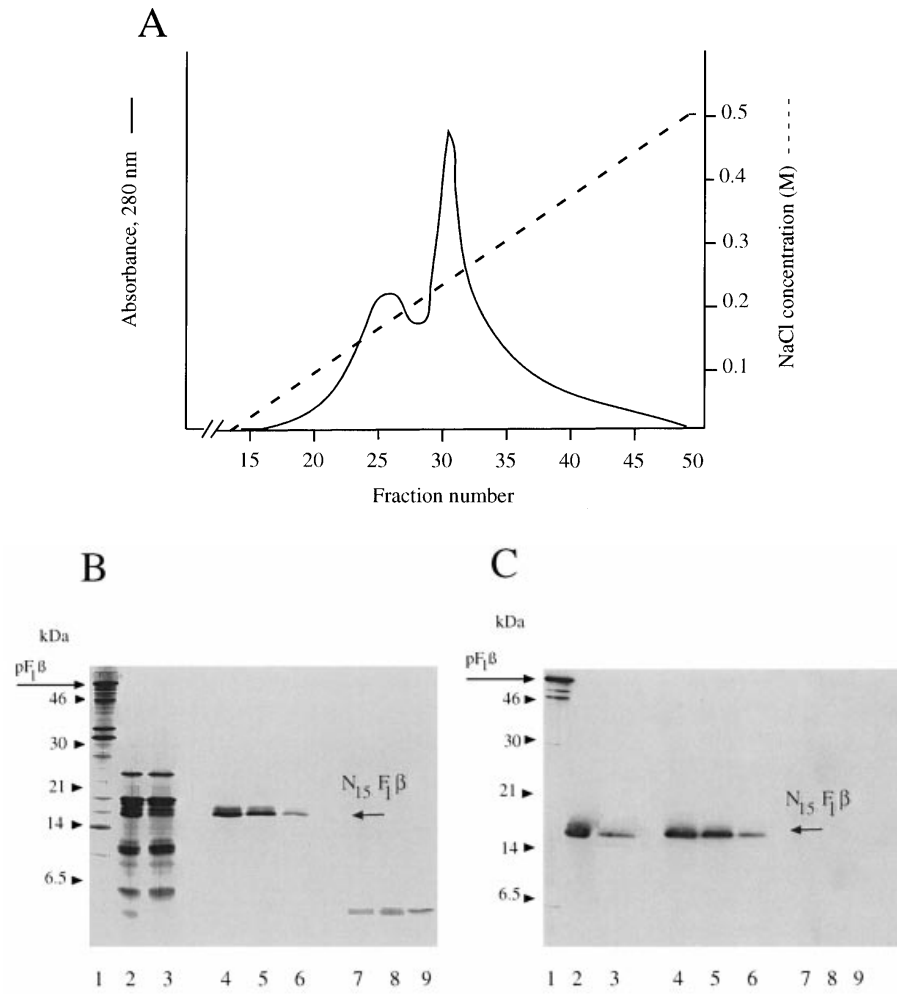

Figure 3 Cleavage of $pF_1\beta$ with CNBr and purification of $N_{15}pF_1\beta$

Purified $pF_1\beta$ was cleaved by CNBr. After cleavage, the peptides were separated on a CM-Sephadex C-25 column. Experimental conditions were as described in the Materials and methods section. The CNBr cleavage fragments and the eluted proteins from the CM-Sephadex C-25 column were analysed by SDS/PAGE [20% (w/v) gel]. (**A**) Elution profile of peptides bound to a CM-Sephadex C-25 column and eluted with a linear gradient of NaCl. (**B**) Analysis of the protein content in fractions after chromatography by silver-staining of the SDS/PAGE gel: lane 1, solubilized inclusion bodies containing $pF_1\beta$ before cleavage with CNBr; lane 2, peptides after cleavage with CNBr and separation on the CM-Sephadex column; lane 3, peptides that were not bound to the CM-Sephadex column; lanes 4–9, fractions after elution with a linear gradient (0–0.5 M) of NaCl. Proteins were revealed by silver-staining. (**C**) Immunological analysis, using antibodies directed against the C-terminal part of the *N. plumbaginifolia* presequence. Lanes are as in (**B**).

0.15–0.25 M NaCl (Figures 3B and 3C, lanes 4–9). N-terminal sequencing of $N_{15}pF_1\beta$ (Ala-Ser-Arg-Arg-Leu-Leu) confirmed our supposition that we obtained the $pF_1\beta$ N-terminal fragment without the first methionine residue, which was cleaved on treatment with CNBr. $N_{15}pF_1\beta$ was concentrated with a Filtron membrane ultrafiltration system (cut-off 3 kDa) to a final protein concentration of 0.4 mg/ml. Finally, from 1 litre of bacterial culture, 0.4 mg of purified $N_{15}pF_1\beta$ was obtained.

Properties of $N_{15}pF_1\beta$

We started our investigation of the properties of $N_{15}pF_1\beta$ by measuring its effect on the membrane potential ($\Delta\Psi$) of isolated potato tuber mitochondria. It was previously shown that the addition of micromolar concentrations of synthetic peptides derived from mitochondrial presequences, or some natural peptides with properties similar to the mitochondrial presequences, resulted in the dissipation of mitochondrial $\Delta\Psi$ [15,26]. Owing to the relatively small size of the N-terminal fragment it was possible that the addition of $N_{15}pF_1\beta$ could result in the dissipation of mitochondrial $\Delta\Psi$. However, at concentrations that completely inhibited the import of other precursors (4 μM), we were not able to detect any decline of $\Delta\Psi$ on the addition of $N_{15}pF_1\beta$ to mitochondria measured as the change in fluorescence quenching of Rhodamine 123 (results not shown). Although the measurement of $\Delta\Psi$ with Rhodamine 123 is not a very sensitive method owing to interaction of the dye with mitochondrial membranes, the method was shown to be accurate at protein concentrations of at most 1 mg/ml [27], and changes of 50 mV could be detected.

To obtain evidence that $N_{15}pF_1\beta$ contains sufficient information to be targeted and processed inside mitochondria we applied different strategies. We investigated direct import and processing of the $N_{15}pF_1\beta$ fragment *in vitro* and its competition with the [^{35}S]methionine-labelled $F_1\beta$ and pAOX synthesized *in vitro* for import and processing.

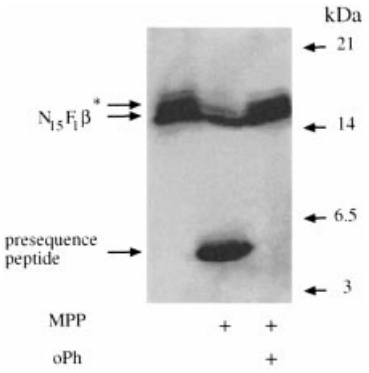

Figure 4 Processing of $N_{15}pF_1\beta$ with isolated spinach MPP–bc_1 complex

Western blot analysis with antibodies directed against the C-terminal part of the *N. plumbaginifolia* $F_1\beta$ presequence. Processing was performed as described in the Materials and methods section. Lane 1, precursor form of $N_{15}pF_1\beta$ (25 nM) (asterisk indicates the 15.5 kDa fragment); lane 2, as lane 1 but in the presence of 0.05 nM MPP–bc_1; lane 3, as lane 2 but in the presence of 5 mM *o*-phenanthroline (oPh).

Antibodies directed to the C-terminal part of the presequence were used for the detection of $N_{15}pF_1\beta$. Figure 4 shows processing *in vitro* of $N_{15}pF_1\beta$ catalysed by the isolated spinach MPP–bc_1 complex. The processing of the precursor fragment(s) of 15 and 15.5 kDa resulted in the generation of a single band of 5.5 kDa corresponding to the presequence containing 53 residues (the presequence minus the N-terminal Met residue). Addition of *o*-phenanthroline, a specific inhibitor of MPP, inhibited processing completely (Figure 4), whereas PMSF had no effect (results not shown). Detection of the processed presequence on import experiments *in vitro* was, however, not possible because of the rapid degradation of the presequence inside mitochondria as evidenced by the addition of the mitochondrial fractions to the processing mixture *in vitro* (results not shown). The addition of PMSF to the import reaction *in vitro* or to the processing assay containing mitochondria failed to prevent the degradation of the presequence peptide (results not shown). Evidence for the direct import of $N_{15}pF_1\beta$ was obtained by using a modified $N_{15}pF_1\beta$, as will be described in the next section.

Preincubation of mitochondria with increasing amounts of $N_{15}pF_1\beta$ resulted in an inhibition of the import of $pF_1\beta$ and pAOX by 95% and 85% respectively (Figure 5). A 50% inhibition of import was achieved with 0.25 μM $N_{15}pF_1\beta$. Binding of $pF_1\beta$ and pAOX to the mitochondrial surface was not affected. Import of $pF_1\beta$ and pAOX into mitoplasts prepared by osmotic rupturing of mitochondrial outer membranes [20] was affected in a similar manner after preincubation of mitoplasts with $N_{15}pF_1\beta$ (results not shown). These results indicate that competition did not occur at the level of the import receptors on the outer membrane but at the level of preprotein insertion and translocation through the mitochondrial import channels or through interaction with other components of the import machinery (e.g. Hsp70 or the MPP–bc_1 complex). We also investigated the effect of $N_{15}pF_1\beta$ on the processing of $pF_1\beta$ and pAOX with the isolated spinach MPP–bc_1 complex. Preincubation of the MPP–bc_1 complex with $N_{15}pF_1\beta$ at micromolar concentrations resulted in the inhibition of processing of $pF_1\beta$ and pAOX synthesized *in vitro* (Figure 6). Half-maximal inhibition occurred at 0.2 and 0.25 μM $N_{15}pF_1\beta$ for the processing of $pF_1\beta$ and pAOX respectively.

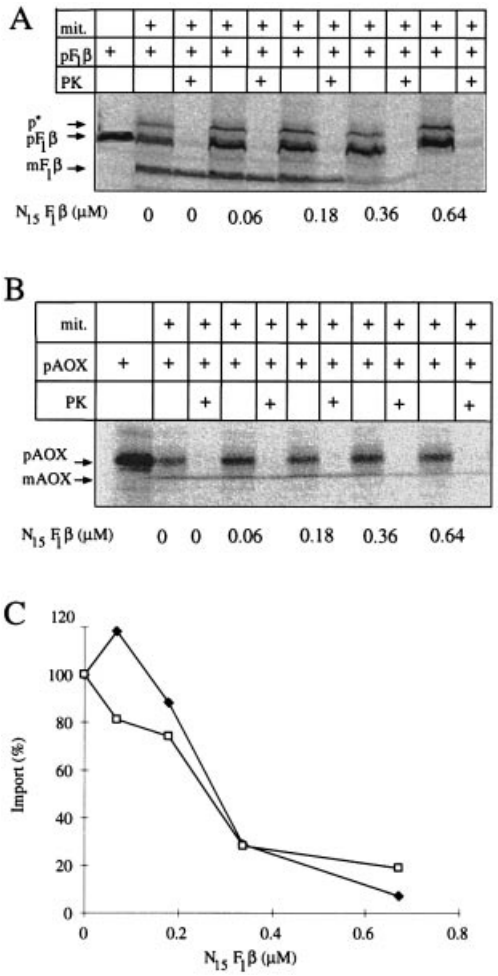

Figure 5 $N_{15}pF_1\beta$ prevents the import of precursor proteins into isolated potato tuber mitochondria

Radiolabelled $pF_1\beta$ and pAOX synthesized *in vitro* were imported into mitochondria as described in the Materials and methods section. Gels after SDS/PAGE were fixed and dried, then scanned with a Fujix BAS 1000 MacBAS Bio-imaging Analyser system. (**A**) Import of $pF_1\beta$ into potato tuber mitochondria. Phosphorimage of the SDS/PAGE of the samples in the absence or the presence of increasing amounts of $N_{15}pF_1\beta$. Abbreviations: p, precursor protein; p*, modified precursor protein; m, mature protein. (**B**) As in (**A**) except that pAOX was used in the import reaction. (**C**) Inhibition profile of import of *N. plumbaginifolia* $pF_1\beta$ (◆) and *Pisum sativum* pAOX (□), synthesized *in vitro*, into potato tuber mitochondria after the addition of $N_{15}pF_1\beta$. The import efficiency was estimated as the percentage of the mature proteinase-K-protected form in lanes in which $N_{15}pF_1\beta$ was present compared to the mature form in the control sample without the addition of peptide.

Preparation of biotin-labelled $N_{15}pF_1\beta$ for import and processing studies *in vitro*

Analysis of the amino acid sequence of the $pF_1\beta$ N-terminal fragment predicts the presence of a single cysteine residue (Cys-93) close to the C-terminus of $N_{15}pF_1\beta$ (Figure 2). The commercial availability of biotin analogues conjugated with maleimide provides the opportunity for labelling substrate proteins with biotin. The results of the biotin-labelling of $N_{15}pF_1\beta$ with MPB are presented in Figure 7. Modification of $N_{15}pF_1\beta$ with MPB resulted in a molecular mass shift of the peptide of approx. 0.5 kDa (Figure 7A). More than 90% of $N_{15}pF_1\beta$ was biotin-labelled. To verify our results, we performed Western blot analysis with avidin conjugated with peroxidase

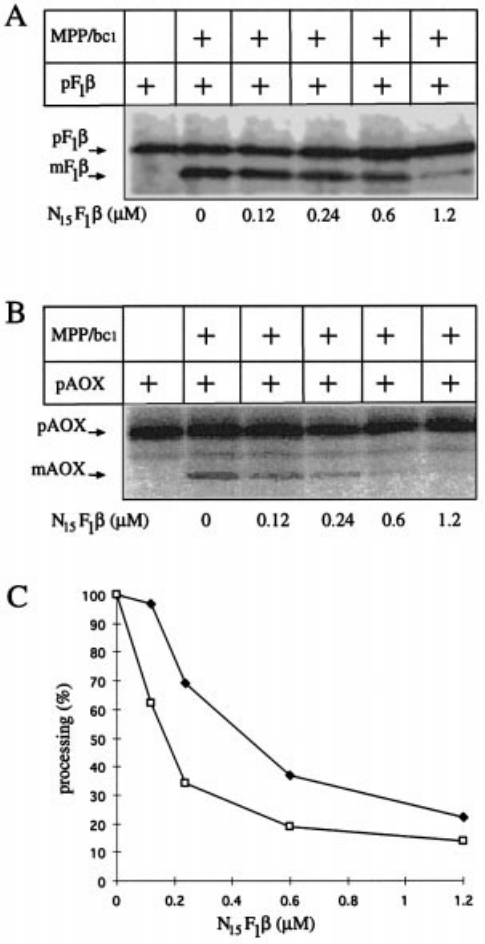

Figure 6 $N_{15}pF_1\beta$ competes with precursors synthesized *in vitro* for processing with the isolated spinach MPP–bc_1 complex

(**A**) Processing of *N. plumbaginifolia* $pF_1\beta$, synthesized *in vitro*, with the isolated MPP–bc_1 complex from spinach. Arrows indicate the positions of precursor (p) and mature (m) proteins. Lane 1, precursor form of the $F_1\beta$ protein; lane 2, processing without the addition of $N_{15}pF_1\beta$; lanes 3–6, processing in the presence of $N_{15}pF_1\beta$. (**B**) Processing of the *Pisum sativum* pAOX, synthesized *in vitro*, with the isolated MPP–bc_1 complex from spinach. Arrows indicate the positions of precursor (p) and mature (m) proteins. Lane 1, pAOX; lane 2, processing without the addition of $N_{15}pF_1\beta$; lanes 3–6, in the presence of $N_{15}pF_1\beta$. (**C**) Inhibition profile of processing of *N. plumbaginifolia* $pF_1\beta$ (◆) and *P. sativum* pAOX (□), synthesized *in vitro*, with the purified MPP–bc_1 complex from spinach after the addition of $N_{15}pF_1\beta$. The degree of inhibition was measured as a ratio of the mature form to the sum of the precursor and mature forms. The efficiency of processing was estimated as described above.

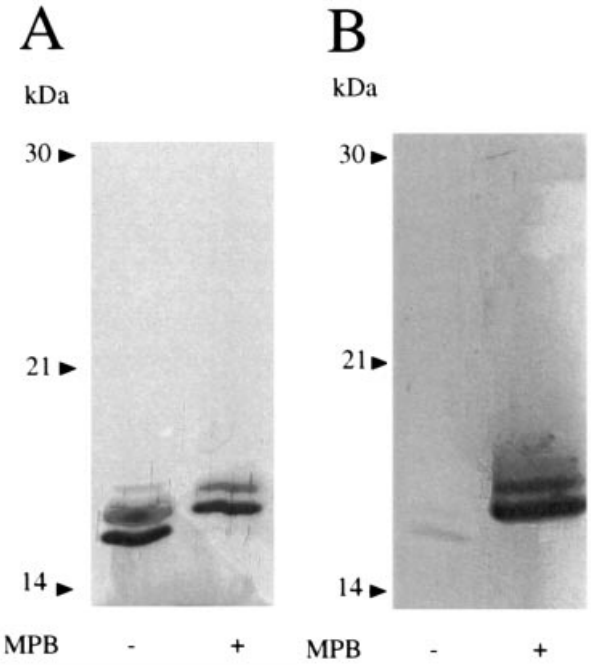

Figure 7 Biotin-labelling of $N_{15}pF_1\beta$ with MPB

(**A**) The biotinylated product was analysed by silver-staining after SDS/PAGE [15% (w/v) gel]. Lane 1, $N_{15}pF_1\beta$; lane 2, biotinylated $N_{15}pF_1\beta$. (**B**) Western blot analysis with avidin–peroxidase. Lanes were as in (**A**).

followed by enhanced chemiluminescence signal development. Our results suggest that only biotin-labelled fragments could be recognized specifically (Figure 7B).

We tested the ability of the biotin-labelled $N_{15}pF_1\beta$ to be imported into mitochondria and to be processed by the isolated MPP–bc_1 complex. Again, we were unable to detect the generation of the processed and/or imported fragments of MPB-labelled $N_{15}pF_1\beta$ in the absence of PMSF; however, in the presence of PMSF a double band of molecular mass approx. 9–9.5 kDa could be seen (Figure 8A). The molecular mass is in agreement with the predicted molecular mass of the mature portion of $N_{15}pF_1\beta$ (82 residues). The addition of thermolysin after completion of the import reaction showed protection of both the precursor and the mature forms inside mitochondria. The protection of precursor forms might be due to the smallness of the fragments, which might be located in the mitochondrial intermembrane space. The addition of valinomycin, a potent inhibitor of protein import, caused an inhibition of the formation of the mature forms and a degradation of MPB-labelled $N_{15}pF_1\beta$ on the addition of thermolysin. Processing *in vitro* of MPB-labelled $N_{15}pF_1\beta$ with MPP resulted in the generation of mature forms of MPB-labelled $N_{15}pF_1\beta$ (Figure 8B). The addition of *o*-phenanthroline to the processing reaction resulted in the inhibition of processing. The processing activity was not sensitive to the addition of PMSF (results not shown). Interestingly, the mature portion of MPB-labelled $N_{15}pF_1\beta$ was less sensitive to degradation than the presequence. The addition of the solubilized mitochondria to the processing reaction did not result in the degradation of mature forms of MPB-labelled $N_{15}pF_1\beta$ (results not shown).

Generation of a translocation intermediate by using an avidin–biotin technique

Another strategy for investigating the import and processing system of mitochondria is the preparation of proteins that can be correctly inserted into the mitochondrial translocation channels and exposed to the matrix but cannot be completely imported owing to the inability of the C-terminal part of the preprotein to be unfolded. The incubation of egg-white avidin with biotin results in the formation of tightly folded tetramers of the avidin–biotin complex [28]. To generate a tightly folded protein complex that could be targeted to mitochondria, we preincubated biotin-labelled $N_{15}pF_1\beta$ with egg-white avidin (see the Materials and methods section). Preincubation of the avidin–biotin-labelled peptide complex with isolated potato tuber mitochondria resulted in the inhibition of protein import of [^{35}S]methionine-labelled

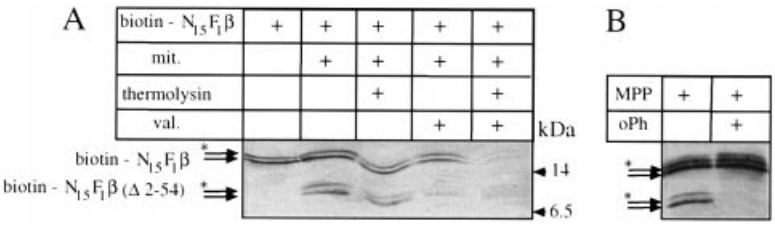

Figure 8 Import and processing of $N_{15}pF_1\beta$–MPB

Western blot analysis was performed with avidin–peroxidase as described in the Materials and methods section. (**A**) Import of $N_{15}pF_1\beta$–MPB into isolated potato tuber mitochondria was performed as described in the Materials and methods section. Arrows indicate the positions of the precursor and mature forms of biotinylated $N_{15}pF_1\beta$ and its 0.5 kDa larger counterpart. (**B**) Lane 1, processing of $N_{15}pF_1\beta$–MPB by isolated spinach MPP–bc_1 complex; lane 2, as lane 1 but in the presence of *o*-phenanthroline (oPh). Arrows as in (**A**).

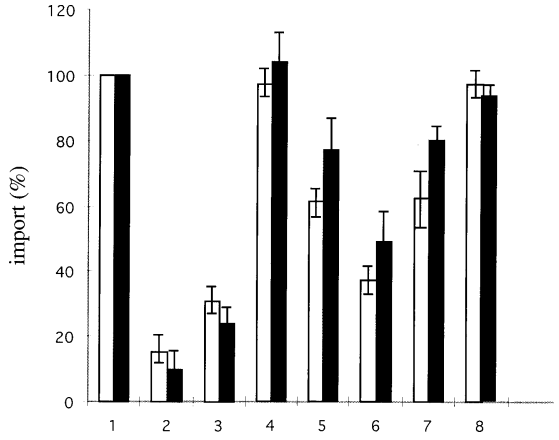

Figure 9 Translocation intermediates accumulated in import sites affect mitochondrial protein import

Import into mitochondria was performed as described in the Materials and methods section. The efficiency of protein import into potato tuber mitochondria was estimated as the amount of mature proteinase-K-protected form. Import into control (without reisolation) mitochondria without additions was taken as 100%. Error bars represent ± S.D. from three experiments. Lane 1, import of $pF_1\beta$ (open column) and pAOX (filled column) into mitochondria, without other additions; lane 2, as lane 1 but with the addition of 0.64 μM $N_{15}pF_1\beta$–MPB to mitochondria 5 min before import; lane 3, as lane 2 but 0.64 μM $N_{15}pF_1\beta$–MPB were mixed with 2.5 μM of avidin in import buffer and added to mitochondria; lane 4, import of $pF_1\beta$ and pAOX into mitochondria that were reisolated twice, without other additions; lane 5, as lane 4 but mitochondria were preincubated with 0.64 μM $N_{15}pF_1\beta$–MPB under import conditions before reisolation; lane 6, as lane 4 but mitochondria were preincubated with 0.64 μM $N_{15}pF_1\beta$–MPB mixed with 2.5 μM of avidin under import conditions before reisolation; lane 7, as lane 6 but 100 μM biotin was added to mitochondria before import of $pF_1\beta$ and pAOX; lane 8, as lane 7 but 2.5 μM avidin was used instead of $N_{15}pF_1\beta$–MPB–avidin mixture.

$pF_1\beta$ and pAOX synthesized *in vitro* (Figure 9, lanes 3 and 6). Reisolation of mitochondria did not result in the dissociation of the precursor–biotin–avidin complex from the translocation channels, in contrast with $N_{15}pF_1\beta$, which only transiently blocked the import pore (Figure 9, lanes 2 and 5). Relatively low rates of inhibition of import of $pF_1\beta$ and pAOX into mitochondria on accumulation of the precursor–avidin complex in import sites (50–60%) might be explained by steric hinderance of the tetrameric precursor–biotin–avidin complex, which is consistent with results reported previously [17]. The addition of excess biotin to the mitochondria with accumulated preprotein–avidin complex in transit resulted in the dissociation of avidin from biotin-labelled $N_{15}pF_1\beta$ and the partial restoration of import of $pF_1\beta$ and pAOX (Figure 9, lane 7). The addition of biotin to mitochondria preincubated with avidin failed to inhibit import (Figure 9, lane 8), indicating that the observed effect was indeed precursor-specific.

DISCUSSION

Here we describe a new strategy for the preparation of chemical quantities of an import-competent precursor for studies of mitochondrial protein import and processing. There have been only a few papers about the purification of chemical amounts of import-competent mitochondrial preproteins overexpressed in *E. coli* either directly in soluble form [29,30] or after solubilization from inclusion bodies [16,17,31]. In most cases, the overexpressed protein is import-incompetent; in some cases the protein remains import-incompetent even after preincubation with 8 M urea or reticulocyte lysate. To obtain an import competent precursor further treatments of precursors are therefore required. In our study we found that chemical cleavage, with CNBr, of $pF_1\beta$ overexpressed in *E. coli* resulted in the generation of an import-competent $N_{15}pF_1\beta$.

The method described here enabled us to overcome the effect of the misfolding of precursors isolated from the inclusion bodies. The method is very efficient and rapid, resulting in the generation of large quantities of pure $N_{15}pF_1\beta$. We tested the proteolytic cleavage of the overexpressed $pF_1\beta$ with glutamic protease V8 under mild conditions but the efficiency of proteolysis was very low. The chemical cleavage with CNBr under denaturing conditions [70% (v/v) formic acid] resulted in a complete degradation of $pF_1\beta$. From 25 mg of inclusion bodies containing 5 mg of $pF_1\beta$ we obtained 0.4 mg of $N_{15}pF_1\beta$, corresponding to a yield of 40%. The purity of the isolated fragment was very high, more than 99%. The purification could be easily achieved owing to the high isoelectric point of the fragment, resulting from the presence of basic residues in the presequence and the absence of acidic residues. The only contaminant was the 15.5 kDa protein, which has 0.5 kDa higher molecular mass than the predicted molecular mass of the fragment with the first methionine residue within the protein (apart from the N-terminal methionine residue). The 15.5 kDa protein has the same N-terminal sequence as $N_{15}pF_1\beta$ and therefore we conclude that it is a product of an incomplete degradation of $pF_1\beta$ at a methionine residue (Met-142) located six residues towards the C-terminus from the first methionine residue (Met-136).

The procedure described has a general value in the isolation of chemical quantities of any mitochondrial presequence peptide or an N-terminal fragment of the precursor protein. The methionine residue can be introduced by site-directed mutagenesis at any location in the precursor protein; the mutant protein can then be overexpressed in *E. coli* and exposed to cleavage with CNBr for

the production of a requested peptide. The methionine residue is not a very frequent amino acid in mitochondrial preseqences, and especially not in plant presequences [32]; the presequence peptide will therefore not normally be subject to internal cleavage. In cases in which a methionine residue occurs in the presequence, it can be replaced by any other non-polar amino acid by site-directed mutagenesis before overexpression. Generally, the pI of mitochondrial presequences is high; presequence peptides can therefore be purified easily by ion-exchange chromatography. The described procedure has many advantages because it overcomes difficulties associated with the insolubility of the overexpressed precursors as well as the great expense of a chemical synthesis of long presequence peptides.

$N_{15}pF_1\beta$ retained all the information necessary for targeting. Our results show that import and processing of the fragment can be achieved; they also indicate that degradation events inside mitochondria have an important role in mitochondrial biogenesis. Because the accumulation of presequence peptides inside mitochondria has a potentially damaging effect on mitochondrial membranes, a rapid degradation of the presequence peptides is assumed to occur; however, the mechanism and regulation of these events are unknown. Non-native or non-assembled imported proteins have been shown to be targets for mitochondrial proteases [33]. Observations that the presequence and the mature portion of $N_{15}pF_1\beta$ have different sensitivities to proteolytic degradation indicate the involvement of different proteases in the degradation of $N_{15}pF_1\beta$.

Competition studies (Figures 5 and 6) indicate that, on its route to mitochondria, $N_{15}pF_1\beta$ competes for import pathways with two other matrix-destined proteins. The competition occurs after the initial steps of the precursor's binding to the surface import receptors, in agreement with previous studies of the effect of synthetic presequence peptides on the import of mitochondrial protein [15,26,34]. Dekker et al. [29] calculated that the concentrations of TOM complexes are 4-fold higher than those of corresponding TIM complexes (on a molar basis) and that the amounts of accumulated translocation intermediates are limited by the number of TIM molecules. Events after the step of protein translocation across the inner mitochondrial membrane have also to be taken into consideration. For example, it has been proposed that there is a direct link between the import and processing events in plant mitochondria [35]. It was shown previously that the MPP recognized some elements of a tertiary structure in the precursor protein [32]. To be processed by MPP as well as to compete with other mitochondrial preproteins in a processing assay *in vitro*, $N_{15}pF_1\beta$ has to adopt the correct conformation. Therefore our results indicate that $N_{15}pF_1\beta$ itself, without the assistance of molecular chaperones, can be refolded at least to some extent.

Different strategies have been used for the generation of mitochondrial targeted preproteins that accumulate at translocation sites spanning both mitochondrial membranes [16,17,36–38]. The C-terminal part of such preproteins can be stabilized in a folded, import-incompetent conformation consisting of an enzyme–substrate complex in which the N-terminal part of such preproteins becomes trapped by the components of TIM machinery [29]. Direct cross-linking between the C-terminal part of a preprotein and a tightly folded passenger protein is also a useful strategy in generating a stable import intermediate [36,37]. The biotinylation of Cys-93 of $N_{15}pF_1\beta$ allows the formation of an avidin–biotin-labelled $N_{15}pF_1\beta$ complex. The accumulation of the translocation intermediate could be modulated by the addition of excess biotin to the reaction system, causing a release of avidin from the biotinylated fragment and allowing translocation to occur. To span both mitochondrial membranes and interact stably with matrix Hsp70, a polypeptide chain must be at least 65 residues in length [39]. On the accumulation of $N_{15}pF_1\beta$ at translocation sites, the presequence, which is 54 residues long, cannot be cleaved off by the matrix-located processing peptidase, which in plant mitochondria is integrated into the bc_1 complex [22]. Translocation intermediates provide a convenient system for studying the properties of mitochondrial import channels and their association with protein import. There is evidence that the TOM and TIM machinery might be linked to membrane channels detectable by electrophysiological experiments. Patch-clamp studies have revealed that at least two types of channel activity are related to the TOM and TIM machinery [40,41]. A peptide-sensitive channel of the yeast outer mitochondrial membrane can be blocked transiently by peptides derived from mitochondrial presequences. TOM40, a major component of the outer membrane translocation channel, was shown to be part of the peptide-sensitive channel [42]. The activity of the multiple conductance channel of the yeast inner membrane was shown to be associated with the TIM complex [41]. Various channel activities have been identified in the mitochondrial membranes of plants and mammals and therefore the generation of translocation intermediates such as the avidin–biotin–$N_{15}pF_1\beta$ complex might be a useful technique for obtaining data on the relationship between import channels and channels detected by patch-clamping experiments.

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