Isolation and identification of a novel mitochondrial metalloprotease (PreP) that degrades targeting presequences in plants

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ABSTRACT

Most of the nuclear encoded mitochondrial precursor proteins contain an N-terminal extension called the presequence that carries targeting information and that is cleaved off after import into mitochondria. The presequences are amphiphilic, positively charged, membrane interacting peptides with a propensity to form $\alpha$-helices. Here, we have investigated the proteolysis of the presequences that have been cleaved off inside mitochondria. A presequence derived from the overexpressed F$_1$F$_\beta$ subunit of the ATP synthase and specific synthetic fluorescent peptides (Pep Tag Protease assay) have been shown to undergo rapid degradation catalyzed by a matrix located protease. We have developed a 3-step chromatographic procedure including affinity and anion exchange chromatography for isolation of the protease from potato tuber mitochondria. Two-dimensional gel electrophoresis of the isolated proteolytically active fraction followed by ESI MS/MS and database searches allowed identification of the presequence peptide degrading protease in Arabidopsis thaliana database as a novel mitochondrial metalloendopeptidase with Mw of 105 kDa. The identified metalloprotease contains an inverted zinc-binding motif and belongs to the pitrilysin family.
INTRODUCTION

Most mitochondrial proteins are encoded by nuclear genes, synthesized on cytosolic polyribosomes as precursor proteins and imported into mitochondria post-translationally. Precursor proteins contain a cleavable peptide extension at the N-terminus, called presequence or targeting or signal peptide (for reviews see (1,2)). The presequences contain information necessary for targeting and are required at all steps along the mitochondrial protein import pathway (3,4). In the cytosol, nascent polypeptide chains and presequences interact with molecular chaperones. Presequences are further recognized by specific receptors on the outer membrane of mitochondria (5,6). The protein import machinery of mitochondria, the translocases of the outer (TOM) and the inner membrane (TIM), contain a series of proteins that successively interact with the presequences (4,7). The mitochondrial Hsp70 is the primary target protein for the presequence protruding into the matrix (8). Finally, the presequence is cleaved off by the mitochondrial processing peptidase (MPP) (9,10) and the mature protein is folded and assembled upon action of molecular chaperones into oligomeric protein complexes.

The great majority of mitochondrial presequences (>80%) are in the range of 20-60 residues, however, the length varies substantially from 6 residues for ATP synthase F chain from yeast to 136 residues for Glycine max cytochrome c oxidase subunit 2 (Cox2) (11,12). Presequences do not show amino acid sequence similarity, but they have characteristic physicochemical properties. They are enriched in positively charged, hydroxylated and hydrophobic residues and have the potential to form an amphiphilic α-helical structure (13,14). Synthetic peptides derived from mitochondrial presequences have been shown to bind to lipid vesicles containing anionic phospholipids as amphiphilic helical structures (15-
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17). The NMR structure of the Tom20 receptor in complex with a presequence peptide revealed that the bound presequence forms an amphiphilic α-helical structure with a hydrophobic surface that interacts with the hydrophobic patch in the Tom20 groove (18).

Several research groups have reported severe effects of presequence peptides on the structural and functional integrity of mitochondrial membranes. Presequence peptides can perturb natural and artificial lipid bilayers (19-21). Addition of presequences to mitochondria results in membrane lysis, uncoupling of respiration and dissipation of the membrane potential (14,22,23). The mechanism of action for membrane permeabilizing peptides is not clear, but it has been proposed that the presequence peptides induce a channel (24) or that the peptides themselves form a pore (25). Furthermore, peptides derived from mitochondrial presequences have been shown to be lethal towards *Bacillus megaterium* at µM concentrations (26). To avoid the potential harmful interactions of presequences with mitochondrial membranes, presequences cleaved off from precursor proteins inside mitochondria should be removed either by degradation inside mitochondria or by export out from the organelle.

Using chemical quantities of the presequence of the ATP synthase F_{1}β subunit we have previously reported experimental evidence for a rapid degradation of the presequence *in organello* after *in vitro* import and processing (27). There are a few reports suggesting that chemically synthesized presequence peptides are degraded inside mitochondria (28,29). The synthetic presequence peptide derived from Cox IV was imported into rat mitochondria and found to be associated with the membrane, however, the authors suggested that the peptide was further translocated to the matrix and degraded over time (29). Another chemically synthesized presequence peptide derived from cytochrome P-450 was accumulated inside mitochondria only in the presence of a chelating agent, o-phenanthroline (28-30). Subunit 9
A novel mitochondrial metalloendopeptidase of the bovine cytochrome $bc_1$ complex corresponds to the presequence of the bovine Rieske iron sulphur protein, and is the only known example of a presequence that is retained in the mitochondria and integrated as a subunit of an oligomeric membrane bound protein complex (31). The precursor of the bovine Rieske iron sulphur protein is targeted and assembled into the $bc_1$ complex before the presequence is cleaved off, presumably by the core proteins in the $bc_1$ complex (31-33).

Several ATP dependent proteases have been identified in mitochondria that selectively remove non-assembled or misfolded polypeptides (34). The quality control of the mitochondrial inner membrane is ensured by the ATP and metal-dependent AAA proteases, Yme1p, Yta10p and Yta12p, homologues of the bacterial FtsH proteases (35). Yme1p, Yta10p and Yta12p have been identified in Neurospora crassa, yeast and mammalian mitochondria (36-38). The PIM1 protease, a homologue of the bacterial Lon protease, is located in the mitochondrial matrix and has overlapping substrate specificities with the AAA proteases (39,40). Lon-like proteases have been found in the mitochondria of yeast and mammals (41,42). Maize mitochondria contain a Lon protease that is loosely associated to the inner membrane (43). Homologues of the bacterial ClpP protease have also been identified in the matrix of mammalian and plant mitochondria (44,45). However, no proteolytic function or natural substrates have been identified for ClpP in mitochondria. Both PIM1 and ClpP are ATP dependent serine type proteases. All the ATP dependent proteases in mitochondria are assembled into large multimeric protein complexes and harbour not only the proteolytic but also chaperone activity (45,46).

Studies from our laboratory revealed that the presequence of $F_1\beta$ undergoes rapid degradation catalyzed by a matrix located protease(s) (27). Here, we have developed a 3-step
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chromatographic procedure for isolation of a protease involved in presequence degradation. The proteolytic activity was measured by immunological detection of the F₁β presequence degradation and by cleavage of synthetic fluorescent peptides. Two-dimensional gel electrophoresis of the isolated fraction followed by ESI MS/MS and database searches allowed identification of the presequence peptide degrading protease in A. thaliana database as a novel mitochondrial metalloprotease. The identified metalloprotease contains an inverted zinc-binding motif and belongs to the pitrilysin family.
EXPERIMENTAL PROCEDURES

Purification of a novel metalloprotease from Solanum tuberosum mitochondria – Potato tuber mitochondria were isolated as described previously (47). The mitochondria were diluted with 20 mM HEPES-KOH (pH 8.0) and 30 mM MgCl₂ to a protein concentration of 8 mg/mL and sonicated for 3 x 15 s at 4°C. Matrix was separated from membranes by centrifugation at 200 000 x g for 30 min. The supernatant was filtered through a 0.2 µm membrane and applied to an Arginine Sepharose (Amersham Biosciences) affinity column equilibrated with 20 mM HEPES-KOH (pH 8.0). Bound proteins were eluted from the column with a linear gradient of 0-0.5 M NaCl. The proteolytically active fractions were pooled, desalted on PD-10 column and loaded onto a Mono Q HR 5/5 (Amersham Biosciences), equilibrated with 20 mM HEPES-KOH (pH 8.0). Bound proteins were eluted from the Mono Q anion exchanger with a linear gradient of 0-0.4 M NaCl. The active fraction was desalted on a PD-10 column and applied to a Mini Q PE 4.6/50 (Amersham Biosciences), equilibrated with 20 mM HEPES-KOH (pH 8.0). Bound proteins were eluted from the Mini Q anion exchanger with a linear gradient of 0-0.4 M NaCl. The active fraction eluted from the Mini Q column was desalted on PD-10 and concentrated.

Two-dimensional gel electrophoresis – Samples containing proteolytic activity (150 µl, 1.8 mg/mL) were diluted to a final volume of 250 µl with a solution containing 7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 100 mM DTT and 2 % (v/v) carrier ampholytes (IPG buffer, pH 3-10 NL Amersham Biosciences) and applied to a non linear IPG strip, pH 3-10. The strips were allowed to rehydrate overnight at 20°C and were then transferred to IPG strip holders and covered with paraffin oil. Proteins were focused for 1 h at 150, 500 and 1 000 V followed by a stepwise increased gradient to 8 000 V. The isoelectric focusing was then completed at a constant voltage of 8 000 V until 80 000 V-h was reached. Subsequently, the strips were first
equilibrated for 15 min in 50 mM Tris-HCl (pH 6.8), 6 M urea, 30 % (v/v) glycerol, 2 % SDS (w/v) and 10 mg/mL DTT and then for 10 min in the same buffer without DTT but with 25 mg/mL iodoacetamide and a trace of bromophenol blue. In the second dimension, 12 % SDS-PAGE was carried out according to Laemmli (48). Proteins were stained with Coomassie Brilliant Blue R-250.

**In-gel digestion, sample preparation, mass spectrometry and data base search** - Protein spots were excised from the Coomassie stained gel and put in a 96-well PCR plate. In-gel digestion was performed according to Lanne et al. (49) with some modifications: gel pieces were washed with water and 35 % acetonitrile followed by dehydration in a SpeedVac vacuum evaporator. The gel plugs were digested with trypsin and extracted by addition of 1 % formic acid/1 % acetonitrile. The digests were then analyzed by MALDI-TOF MS on a Voyager-DE STR mass spectrometer in reflector mode (Perseptive Biosystems) with alfa-cyano-4-hydroxycinnamic acid as matrix. To achieve accurate mass determination, calibration was automatically performed using tryptic auto-digestion products as internal standards for each sample. The obtained mass spectra were annotated in Data Explorer and the latest versions of NCBI nonredundant databases were searched with the resulting peptide mass list, using the search engine MASCOT (http://www.matrixscience.com) or Profound (http://prowl.rockefeller.edu/cgi-bin/ProFound). Remaining peptide extracts were purified and concentrated on a column of Poros™ 20R packing material (Applied Biosystems). About 5 µl of sample was applied onto the column, washed with 5 % formic acid and the peptides were eluted with 2.5 µl 65 % acetonitrile and 0.5 % formic acid directly into the nanospray needle (Protana A/S). The samples were analyzed by electrospray MS using a Q-TOF mass spectrometer (Micromass) equipped with a nanospray interface. The MS/MS spectra were
either used to search NCBI nonredundant databases with MASCOT directly or interpreted manually using MassLynx and Pepseq (Micromass).

**Substrate preparations** - The precursor of the F1β subunit of the mitochondrial ATP synthase, pF1β, from *N. plumbaginifolia* was overexpressed in *E. coli* BL21(DE3). The expressed protein was insoluble and present in inclusion bodies. Isolated inclusion bodies were solubilized in 70% formic acid and chemically cleaved with CNBr after methionine residues. The sample was evaporated and redissolved in 10 mM Tris-HCl (pH 8.0), 4 M urea, 1 mM DTT and loaded onto a Mini S column. Elution was performed with a linear gradient of 0-0.5 M NaCl. Fractions containing the 15 kDa N-terminal fragment of pF1β, N15pF1β, were collected. This polypeptide contains all information necessary for mitochondrial targeting, import and processing (47). N15pF1β (0.01 nM) was cleaved with purified MPP/bc1 complex from *Spinacia oleracea* to generate the presequence of N15pF1β. The presequence is 54 amino acid residues long, has a molecular mass of 5.7 kDa and is referred to as N5.7pF1β(2-54) (27).

The presequence with a modified C-terminal carboxylic group was prepared from a mutant pF1β, in which a methionine was inserted at position +1 downstream of the MPP processing site. The mutant protein was overexpressed in *E. coli* and treated with CNBr to cleave the presequence after the introduced methionine. During the cleavage reaction the methionine was converted into a homoserine lactone (hsl) group to generate N5.7pF1β(2-54)-hsl (50).

Purification of N5.7pF1β(2-54)-hsl was performed as described above for N15pF1β.

**Protease activity** – The proteolytic activity was determined by degradation of N5.7pF1β(2-54) that was generated by cleavage of N15pF1β with MPP/bc1 complex. For the proteolytic reaction, precleaved N15pF1β (0.01 nM) was incubated with mitochondrial matrix (33 µg) or...
isolated chromatographic fractions (30 µl) in a buffer containing 20 mM HEPES-KOH (pH 8.0), 1 mM MnCl₂ and 1 % (v/v) Triton X-100 for 30 min at 30°C. The reaction was stopped by addition of sample buffer and analyzed on 12–20 % SDS-PAGE in the presence of 4 M urea (48). Immunological cross-reactivity was analyzed by Western blotting using Hybond ECL that was immunodecorated with antibodies raised against the C-terminal part of the presequence (residue 38-54) of the pF₁β, followed by detection with horseradish peroxidase labeled secondary antibodies. Degradation of N₅.₇pF₁β(2-54)-hsl was studied to assess the role of the free C-terminus in proteolysis. The degradation assay contained N₅.₇pF₁β(2-54)-hsl (0.01 nM) and the isolated fraction from the Mini Q column (30µl) and was carried out for 30 min at 30°C. The reaction was stopped and products were analyzed as described for the N₅.₇pF₁β(2-54). To characterize the protease, degradation of synthetic peptides was also studied. The degradation assay contained 0.6 µg of the synthetic fluorescent peptides, P₁ or P₂ (Pep Tag Protease assay, SDS-Promega), and the isolated fraction from the Mini Q column (30µl). Degradation was carried out at 30°C for 30 min. 80 % glycerol was added and the samples were analyzed directly on a 1 % agarose gel and the fluorescent peptides were visualized by UV-light. The effect of protease inhibitors and ATP dependency of the proteolytic activity was investigated using 1 mM PMSF, 1 mM NEM, 10 mM orthophenanthroline, 1 mM carboxypeptidase inhibitor (Calbiochem 217359) or 40 U/mL apyrase. The isolated fraction from the Mini Q column was preincubated for 10 min at 4°C with protease inhibitors, before addition of substrates. Both N₅.₇pF₁β(2-54) and the P₁ peptide were used as substrates. To test proteolytic activity in the presence of inhibitors, samples were incubated and analyzed as described before.
**Protein content** - The polypeptide content of the mitochondrial matrix and isolated chromatographic fractions was analyzed by 12-20 % SDS–PAGE in the presence of 4 M urea (48). Proteins were stained with silver.

**RESULTS**

**Purification of a mitochondrial protease that degrades mitochondrial presequences** - In order to assay degradation of mitochondrial presequences we have prepared chemical amounts of a truncated precursor derived from the overexpressed full-length precursor of the F1β (pF1β) subunit of the ATP synthase. The 15 kDa N-terminal fragment of pF1β, designated as N15pF1β, consisted of a 53 amino acid long presequence and the first 82 amino acids of the mature form, and was detected by antibodies directed against the C-terminal part of the presequence (47). The precursor protein was incubated with the isolated spinach MPP/bc1 complex (51) to generate the presequence peptide of 5.7 kDa, N5.7pF1β(2-54) (Fig. 1B, lane 1). The proteolytic activity was found to be localized to the matrix of mitochondria (27). We have developed a 3-step procedure for purification of a protease involved in presequence degradation. The procedure was based on affinity chromatography, followed by anion-exchange chromatography. Mitochondrial matrix from *S. tuberosum* was applied to an affinity column, Arginine Sepharose. The majority of proteins did not bind to the column (flow-through, FT). Bound proteins were eluted with a linear gradient of 0-0.5 M NaCl and recovered in three protein peaks, A1 and A2 at 0.1-0.25 M NaCl and A3 at 0.25-0.35 M NaCl (Fig. 1A). When the isolated fractions were analyzed for proteolytic activity using N5.7pF1β(2-54) (Fig. 1B), the proteolytic activity was found in fractions A1 and A2 (Fig. 1B, lanes 4 and 5). The protein content of eluted fractions analyzed by SDS-PAGE revealed a similar polypeptide pattern in fractions A1 and A2 (not shown) that were pooled, desalted
A novel mitochondrial metalloendopeptidase and loaded onto a Mono Q column. Proteins eluted from the Mono Q with a linear gradient of 0-0.4 M NaCl, (Fig. 2A, upper panel) exhibited two protein peaks, MoQ1 and MoQ2. The proteolytic activity was eluted at 225-275 mM NaCl in fraction MoQ1 (Fig. 2B, lane 3). SDS-PAGE of MoQ1 revealed a major band of 23 kDa as well as several other bands in the high molecular mass region (Fig. 2C). MALDI-TOF analysis identified the 23 kDa as Mn$^{2+}$ containing superoxide dismutase (Mn-SOD). In order to separate Mn-SOD from potential protease candidates, an anion exchanger, Mini Q, with a higher resolution capacity than Mono Q was used in the purification procedure. Proteins bound to the Mini Q were eluted with a linear gradient of 0-0.4 M NaCl and collected in two protein fractions MiQ1 and MiQ2 (Fig. 2A, lower panel). The proteolytic activity was completely eluted at 310-340 mM NaCl and found only in fraction MiQ2 (Fig. 2C, lane 6). As revealed by SDS-PAGE in one dimension, fraction MiQ1 contained the 23 kDa Mn-SOD band (Fig. 2D), whereas MiQ2 contained 7 polypeptides: 2 high molecular mass proteins (110 kDa and 108 kDa), 4 proteins in the range of 55-66 kDa (66 kDa, 65 kDa, 55 kDa and 54 kDa) and residual amounts of Mn-SOD (23 kDa) (Fig. 2C). No proteolytic fragments of the presequence of pF1$\beta$ were detected after incubation with isolated chromatographic fractions (Fig. 1B, lanes 4 and 5; Fig. 2 lanes 3 and 6). In order to confirm complete degradation of the presequence we used N$_{5:7}$pF1$\beta$(2-54) that was labeled with sulpho succinimidyl biotin (SSB) at lysine residues and detected using the avidin peroxidase system (27). There are 2 lysine residues in the presequence of F1$\beta$ at positions 32 and 44. Experiments with the SSB-labeled F1$\beta$ presequence have not revealed any presequence fragments indicating complete degradation of the presequence (not shown).

Identification of the protease: two-dimensional gels and mass spectrometry - To identify the protease among the polypeptides in the MiQ2 fraction, two-dimensional gels followed by MALDI-TOF and ESI-MS/MS was applied. The two-dimensional gel of the MiQ2 fraction
revealed 9 individual protein spots; two high molecular weight proteins (105 kDa and 91 kDa), 5 proteins in the range of 55-70 kDa, a 35 kDa protein and Mn-SOD (23 kDa) (Fig. 3). The molecular masses of proteins separated in one or two dimensions differ slightly from each other. This is due to the presence of urea in the one dimensional SDS-PAGE system. MALDI-TOF followed by database search allowed identification of the proteins in the 55-70 kDa range as the F1α and F1β subunits of ATP synthase (54 and 55 kDa), the flavoprotein from succinate dehydrogenase (66 kDa), dihydrolipoamide dehydrogenase (57 kDa) and mitochondrial Hsp70 (69 kDa) (Table 1). The F1α and F1β subunits are a part of the extra membranous catalytic core of the membrane bound ATP synthase (52). Western blot analysis revealed small contaminations of the F1α and F1β subunits in the matrix preparations, <5%. Both the flavoprotein from succinate dehydrogenase and dihydrolipoamide dehydrogenase have been classified as soluble matrix proteins in studies of A. thaliana mitochondrial proteome (53). Hsp70 is one of the most conserved proteins and described as a general molecular chaperone (54). In mitochondria, Hsp70 is localized to the matrix and drives mitochondrial import in an ATP dependent manner (55). Mn-SOD has been identified in the matrix of mitochondria (53) and functions to neutralize produced oxygen radicals into H2O2 (56). All the proteins identified by MALDI-TOF analysis are conserved, abundant mitochondrial proteins with a well-characterized function. The 3D structures have been solved for the MALDI-TOF identified proteins or their protein homologues and no proteolytic sites or activities have been reported, therefore it allows to exclude them as protease candidates (56-61). No MALDI-TOF spectra could be obtained from spot nr 9, neither could this spot be stained with silver.

In order to identify the 105 kDa and 91 kDa proteins we performed ESI-MS/MS analysis to obtain sequence information in combination with molecular masses. ESI-MS/MS identified...
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the 91 kDa protein as a putative Hsp90 from *A. thaliana* (Table 1). Subcellular prediction programs identified the putative Hsp90 as a mitochondrial protein. Hsp90 has also been found in human mitochondria (62). No proteolytic activity has been reported for Hsp90. The 18 amino acid residues sequence of the peptide derived from the 105 kDa protein (Fig. 4, Table 1) matched four proteins in the NCBI database, a zinc metalloprotease (insulinase family) (BAB02957), a putative zinc metalloprotease (AAG13049) and two putative hydrogenases (AAL67002 and NP_175386). The sequences of the putative zinc metalloprotease and the putative hydrogenases display 99% sequence identity and only differ in a stretch of 10 amino acids located in position 189-199 from the predicted cleavage site. These protein sequences are derived from the same gene (At1g49630) on chromosome 1 and therefore refer to the same protein reported three times in the NCBI databases that will be referred to here as a putative zinc metalloprotease. The gene for the zinc metalloprotease (At3g19170) is located on chromosome 3. The putative zinc metalloprotease and the zinc metalloprotease display 89% sequence identity and 95% sequence similarity (Fig. 4), with most differences in the predicted targeting sequences. The targeting peptide of zinc metalloprotease is 30 amino acids shorter than the targeting peptide of the putative zinc metalloprotease. However, an EST clone of zinc metalloprotease from *A. thaliana* containing an additional 30 amino acid N-terminal region has been reported from the RIKEN Genomic Science Center, Japan and Kazusa DNA Research Institute, Japan. The overexpressed *A. thaliana* zinc metalloprotease revealed proteolytic activity against presequence peptide. Using MitoProt, both proteases are predicted to contain a mitochondrial presequence with an arginine in position –2 upstream of the processing site, a common characteristic of mitochondrial presequences. However, using prediction programs that select preferable organellar localization, Predator and TargetP, the zinc metalloprotease with the additional 30

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A novel mitochondrial metalloendopeptidase amino acid N-terminal region is predicted to be localized to mitochondria whereas the putative metalloprotease was suggested to be a chloroplastic protein. The inverted zinc-binding region is located in the N-terminal portion of the protein and classifies the proteases to the pitrilysin family. We have named the experimentally identified metalloprotease that degrades targeting sequences as Presequence Protease, PreP.

Characterization of PreP, a novel mitochondrial metalloprotease - We investigated if the purified protease was capable of degrading substrates other than the F1β presequence. For this purpose we used synthetic, cleavage specific peptides, P1 and P2, labeled with a fluorescent dye. Both peptides contained typical presequence elements like basic and hydrophobic residues but no acidic residues (Fig. 5A). In the native state both peptides are positively charged (Fig. 5B, C lane 1) but upon cleavage, fragments of different sizes and charges are generated that correspond to different cleavage sites. Incubation of the fraction containing PreP and the P1 peptide produced a negative charged fragment, indicating that the P1 peptide was cleaved after Pro, Leu or Ser (Fig. 5B lane 2). In contrast, the P2 peptide was not degraded by the PreP containing fraction (Fig. 5C, lane 2). These results may reflect the requirement of a minimal peptide length or amino acid content to initiate and pursue degradation.

The effect of protease inhibitors on degradation of N5.7pF1β(2-54) and synthetic peptides by PreP was evaluated (Fig. 6). Degradation of N5.7pF1β(2-54) was inhibited by o-phenanthroline and carboxypeptidase inhibitor (Fig. 6A, lanes 5 and 6). The same protease inhibitors also abolished degradation of the P1 peptide (Fig. 6B, lanes 5 and 6). No effect was observed with PMSF, NEM or apyrase. In order to determine if a free carboxyl group on presequences is required for their degradation we used a presequence with a blocked carboxyl
A novel mitochondrial metalloendopeptidase group, N$_{5.7}$F$_1$$\beta$(2-54)-hsl. Despite the modified C-terminus, N$_{5.7}$F$_1$$\beta$(2-54)-hsl was degraded by the PreP containing fraction (Fig. 7). The carboxypeptidase inhibitor is a 38 amino acid residue peptide isolated from potato that contains presequence characteristic amino acids, 5 positively charged and 11 hydrophobic amino acids (63). Therefore, the carboxypeptidase inhibitor peptide might function as a competitive substrate for the protease.
DISCUSSION

Mitochondrial targeting presequences are potentially harmful to the structure and function of mitochondria as they can disrupt biological membranes, dissipate membrane potential and cause severe mitochondrial dysfunctions (19,21,22). Therefore, it is generally believed that after protein import and processing, the presequences are degraded inside mitochondria. However, virtually nothing is known about the degradation process or proteases involved in the degradation. Difficulties studying the fate of targeting peptides have come from the fact that small quantities of in vitro transcribed and translated precursors have been used in protein import studies. We have used chemical quantities of a precursor protein and we were able for the first time to visualize degradation of a presequence inside mitochondria. We have shown that the presequence of the F1β subunit of the ATP synthase was rapidly degraded by a matrix located protease(s) (27).

The current investigation aimed to identify and characterize mitochondrial protease(s) involved in presequence degradation. We have developed a simple chromatographic procedure for the isolation of the protease(s). The first step of the procedure included purification on an affinity column that contained arginines coupled to Sepharose (Arginine Sepharose). Since mitochondrial presequences are enriched in positively charged residues, arginines and lysines, the idea was to use a positively charged chromatographic gel matrix to bind potential protease candidates that would bind to presequences, in situ. Further purification was achieved by ion-exchange chromatography, on Mono Q and high-resolution Mini Q anion exchangers. The proteolytic activity of the isolated fraction was enriched by several hundred folds in comparison to the matrix. The two-dimensional gel of the purified proteolytically active fraction from the Mini Q column revealed 9 individual proteins, six out
of which were identified by MALDI-TOF as mitochondrial Hsp70, flavoprotein of succinate dehydrogenase, dihydrolipoamide dehydrogenase, the $F_1\alpha$ and $F_1\beta$ subunits of the ATP synthase and Mn-SOD. These proteins were excluded as protease candidates since they have well characterized function and structures with no indication of a proteolytic catalytic site. The 35 kDa protein could not be identified by any means. It was visualized with Coomassie blue, but not with silver staining and no MALDI-TOF spectra could be obtained. These results may indicate a non-proteinaceous nature of this spot. Two residual spots were identified by ESI-MS/MS in order to obtain sequence information in combination with molecular masses. ESI-MS/MS identified the 91 kDa protein as a putative Hsp90 molecular chaperone, whereas the 105 kDa protein was identified as a metalloprotease in the $A.\ thaliana$ databases. The identified metalloprotease harbourd an N-terminal mitochondrial targeting sequence, suggesting that it is localized to mitochondria. The metalloprotease contained an inverted zinc-binding motif (HILEHX$_{74}$E) and belongs to the pitrilysin family. Members of the pitrilysin family can be divided into the pitrilysin subfamily and the mitochondrial processing peptidase subfamily. The identified metalloprotease exhibits high overall homology with proteases of the pitrilysin subfamily. Members of the pitrilysin family are oligopeptidases of 100 kDa, such as insulinase from mammals and its homologue in bacteria, protease III (64). In the cell, these peptidases degrade small peptides of the similar size as mitochondrial presequences (65). Interestingly, insulinase was also found to degrade the cleaved leader peptide present in a peroxisomal protein (66).

Homologues of the zinc metalloprotease are present in human and in yeast. Subcellular prediction programs suggest that both homologues are localized to mitochondria. Alignments of the $A.\ thaliana$ zinc metalloprotease and non-plant eucaryotic homologues revealed 30% sequence identity and a fully conserved inverted zinc-binding motif. The human homologue
of 110 kDa (hMP1) was found to be sensitive to metal chelators and thiol reagents, and was highly expressed in mitochondrial enriched tissues such as heart and skeletal muscles (67). The homologue identified in yeast is a hypothetical 989 amino acids long protein (Ydr430cp). In yeast gene deletion studies, Ydr430cp was not found to be essential (68). No lethal phenotypes have been reported for mitochondrial proteases or their bacterial homologues (46,68,69). The absence of severe phenotypes for mitochondrial proteases suggests overlapping substrate specificities between the proteases, shown e.g. for the PIM1 and the m-AAA proteases (40). The identification of the zinc metalloprotease homologues in various types of mitochondria might indicate that it is a general protease involved in the degradation of cleaved presequences peptides inside mitochondria.

The mitochondrial ATP dependent proteases with different sub-organellar locations generate oligopeptides upon catalysis. Proteolysis by the membrane bound AAA proteases generates small peptides with molecular masses of 2 100 to 600 Da (70). In the matrix, the PIM1 protease degrades proteins to products containing 5-15 amino acid residues (71). In *E. coli*, ClpP hydrolyses a number of proteins by endoproteolysis to generate short acid-soluble peptides (69). ClpP has been identified in the matrix of mitochondria, however, no native substrates or ClpP proteolytic activity have been demonstrated (44,45). Incubation of PreP with the presequence of F₁β revealed no proteolytic fragments. It was evidenced both upon immunodecoration of N₅₋₇pF₁β(2-54) with antibodies directed against its C-terminal part or when the N₁₅pF₁β was labeled with SSB at lysines residues for detection with avidin peroxidase (27). It has been reported that cleavage of chloroplastic transit peptides in the stroma by SPP generates trimmed peptide fragments (72).
Further characterization of PreP was performed using synthetic cleavage-specific fluorescent peptides, P1 (11 aa) and P2 (7 aa). Like mitochondrial presequences, the peptides contained basic and hydrophobic residues and no negatively charged residues. Only the longer P1 peptide was degraded by PreP. This indicates that a minimal peptide length or specific amino acids might be required for efficient proteolysis by the PreP protease. The exact prerequisites for proteolysis still need to be determined. The proteolytic activity was investigated in the presence of inhibitors affecting different protease classes such as metallo, carboxy, serine and cysteine proteases. The chelator o-phenanthroline completely inhibited the degradation of the F1β presequence and the P1 peptide, demonstrating the need of metals for proteolysis. A partial inhibition of the activity was seen with a carboxypeptidase inhibitor, however, the presequence with a modified C-terminal carboxyl group was completely degraded by PreP indicating that there was no requirement of a free carboxyl terminus for degradation. The carboxypeptidase inhibitor is a peptide of 38 amino acids residues purified from potato and can thus function as a competitive substrate in presequence degradation. Classical protease inhibitors affecting serine and cysteine proteases did not abolish the activity. No ATP dependency was shown for PreP. Most proteases in the cell cleave substrates in an ATP independent manner. ATP is not required for peptide bond hydrolysis per se but rather for unfolding or remodeling target substrates. The lack of ATP dependency for presequence degradation might be due to the fact that cleaved presequences are short, soluble peptides without structure in an aqueous environment (15). In conclusion, we characterize PreP as a metalloendopeptidase.

Can presequences be exported from mitochondria? A signal peptide was found to be exported from the ER-membrane and interacted with calmodulin (73). The binding of signal peptides to calmodulin suggests that signal peptides contribute in regulation of cellular reactions (73).
In yeast the ATP-binding cassette (ABC) protein Mdl1 was shown to be involved in export of peptide fragments generated by proteolysis of the inner membrane AAA proteases (70). However, the majority (70%) of the released products from mitochondria were recovered as free methionine (70). Due to high energy costs and low export efficiency of peptides to the outside of mitochondria, presequences are more likely to be degraded inside mitochondria. Amino acids recovered from the degraded presequences can be used for mitochondrial protein synthesis or further catabolised via the urea cycle. Mitochondrial presequences are enriched in arginines that can be a precursor for synthesis of nitric oxide, polyamines and other biologically active compounds. The arginine converting enzymes like arginase and nitric oxide synthase have been identified in mitochondria (74,75). Arginines that are released upon presequence degradation can be converted into nitric oxide and regulate transcription of nuclear encoded mitochondrial genes, thereby providing cross talk between mitochondria and the nucleus.
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FIGURE LEGENDS

FIG. 1. Arginine Sepharose chromatography. A, elution profile from Arginine Sepharose. Mitochondrial matrix was prepared from *S. tuberosum* and applied to the column. Unbound proteins were removed by washing with 20 mM HEPES-KOH, pH 8.0 (flow-trough, FT). Bound proteins were eluted from the affinity column with a linear gradient of 0-0.5 M NaCl (fractions A1-A3). The solid line represents the absorbance at 280 nm and the dashed line, the NaCl gradient. B, degradation of the presequence of F1β (N5.7pF1β(2-54)) with fractions from Arginine Sepharose chromatography. N5.7pF1β(2-54) was obtained after cleavage of N15pF1β with the isolated MPP/**bc1** complex (lane 1) and further incubated with matrix (lane 2), FT (lane 3) or fractions A1, A2 and A3 (lanes 4-6) as described in ”Experimental Procedures”.

FIG. 2. Ion exchange chromatography. A, elution profile from Mono Q (upper panel) and Mini Q (lower panel). Active fractions from Arginine Sepharose were applied to Mono Q. Bound proteins were eluted from the anion-exchanger with a linear gradient of 0-0.4 M NaCl (fractions MoQ1 and MoQ2). The proteolytically active fraction from Mono Q was desalted and loaded onto Mini Q. Bound proteins were eluted from the anion exchanger with a linear gradient of 0-0.4 M NaCl (fractions MiQ1 and MiQ2). The solid line represents the absorbance at 280 nm and the dashed line, the NaCl gradient. B, degradation of the presequence of pF1β, N5.7pF1β(2-54) with fractions from ion exchange chromatography. N5.7pF1β(2-54) was obtained after cleavage of N15pF1β with MPP/**bc1** (lane 1) and further incubated with matrix (lane 2) or fractions MoQ1 (lane 3), MoQ2 (lane 4), MiQ1 (lane 5) and MiQ2 (lane 6) as described in ”Experimental Procedures”. C, SDS-PAGE of fractions eluted from anion exchangers. Proteins were stained with silver.
FIG. 3. **Two-dimensional gel electrophoresis.** The proteins in fraction MiQ2 were resolved by SDS electrophoresis in a 12 % polyacrylamide gel subsequent to isoelectric focusing in a nonlinear immobilized pH gradient from pH 3 to 10.

FIG. 4. **Sequence alignment of the zinc metalloprotease (insulinase family) (BAB02957), a putative zinc metalloprotease (AAG13049) and two putative hydrogenases (AAL67002 and NP_175386) from A. thaliana.** The completely conserved residues are colored in blue, and the conserved residues that are similar are colored in green. The arrow indicates the processing site predicted by Mitoprot. The inverted zinc-binding region, HILEHX74E, is colored in red and the peptide identified by ESI MS/MS is colored in yellow. Multiple sequence alignments were done using ClustalW.

FIG. 5. **Degradation of synthetic fluorescent peptides by PreP containing fraction.** A, sequences of synthetic fluorescent peptides P1 and P2. B, P1 peptide alone (lane 1). P1 after incubation with PreP containing fraction (MiQ2) (lane 2) as described in ”Experimental Procedures”. C, P2 peptide alone (lane 1). P2 after incubation with PreP containing fraction (lane 2) as described under ”Experimental Procedures”.

FIG. 6. **Effect of inhibitors on the PreP activity.** A, degradation of the presequence of pF1β, N5,pF1β(2-54) by PreP containing fraction (MiQ2) in the presence of inhibitors. N5,pF1β(2-54) were obtained after cleavage of N15pF1β with MPP/bc1 complex (lane 1) and further incubated with fraction MiQ2 (lane 2) or fraction MiQ2 in the presence of inhibitors (lanes 3-7) as described under ”Experimental procedures”. B, degradation of the P1 peptide by PreP containing fraction in the presence of inhibitors. P1 peptide alone (lane 1). P1 after incubation
with fraction MiQ2 (lane 2) or with fraction MiQ2 in the presence of inhibitors (lane 3-7) as described in "Experimental Procedures". Cpi: carboxypeptidase inhibitor, o-ph: orthophenanthroline.

FIG. 7. 
PreP catalyzed degradation of the N\textsubscript{S,7}pF\textsubscript{1}\beta(2-54)-hsl presequence that has a modified C-terminus. A, N\textsubscript{S,7}pF\textsubscript{1}\beta(2-54)-hsl alone (lane 1). N\textsubscript{S,7}pF\textsubscript{1}\beta(2-54)-hsl after incubation with PreP containing fraction (MiQ2) as described in "Experimental procedures" (lane 2).

Table I. Proteins identified by mass spectrometry. Proteins were identified by a combination of MALDI-TOF and ESI MS/MS. The spot numbers as indicated in the 2D gel in Figure 4.
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## Table 1

| Spot no | Protein name | Accession no | Organism | Identification | Mass exp./theor. | pI exp./theor. | Experimental sequence |
|---------|--------------|--------------|----------|---------------|-----------------|---------------|-----------------------|
| 1       | Succinate dehydrogenase flavoprotein α-subunit | NP_201477 | A. thaliana | MALDI | 66/70 | 5.8/5.9 | ND |
| 2a      | Dihydrolipoamide dehydrogenase | AAG17888 | S. tuberosum | MALDI | 57/54 | 6.0/6.4 | ND |
| 2b      | Dihydrolipoamide dehydrogenase | AAG17888 | S. tuberosum | MALDI | 61/54 | 6.3/6.4 | ND |
| 2c      | Dihydrolipoamide dehydrogenase | AAG17888 | S. tuberosum | MALDI | 61/54 | 6.4/6.4 | ND |
| 3       | ATP synthase α-chain | P05493 | P. sativum | MALDI | 56/55 | 6.0/6.0 | ND |
| 4       | Superoxide dismutase | P11796 | N. plumbag. | MALDI | 23/25 | 6.1/7.9 | ND |
| 5       | ATP synthase β-chain | P17614 | N. plumbag. | MALDI | 55/60 | 5.4/6.0 | ND |
| 6       | Heat shock 70kDa protein | Q08276 | S. tuberosum | MALDI | 69/73 | 5.6/6.4 | ND |
| 7       | Putative heat shock 90 kDa protein | NP_187434 | A. thaliana | ESI-MS/MS | 91/91 | 5.1/5.3 | LMDLIVNSLYSNK IYEMXXXXLXGK |
| 8       | Putative zinc metalloprotease | AAG13049 | A. thaliana | ESI-MS/MS | 105/121 | 5.1/5.4 | AIIGTIGDVDSYQLPDAK |
| 9       | Putative zinc metalloprotease | BAB02957 | A. thaliana | ESI-MS/MS | 105/117 | 5.1/5.3 | AIIGTIGDVDSYQLPDAK |
| 10      | Putative hydrogenase | AAL67002 | A. thaliana | ESI-MS/MS | 105/117 | 5.1/5.3 | AIIGTIGDVDSYQLPDAK |

**a** Accession number in NCBI databases  
**b** Experimental (kDa)/ theoretical mass of precursor form (kDa)  
**c** Experimental isoelectric point / theoretical isoelectric point of precursor form  
**d** Peptide sequence of the precursor ion; ND, not determined
Figure 1
Figure 2
AAG13049  DRTCYPVASTNKKDFYNLVDVYLDAVFFPKCVDDVHTFQQEGWHYELNDPSEDISTKVFGIVFRTPPKDSTGIPHELIVERLTVLGCGRKYPVKEPFVELLKGSLHTFLNAFTYP
AAL67002  DRTCYPVASTNKKDFYNLVDVYLDAVFFPKCVDDVHTFQQEGWHYELNDPSEDISTKVFGIVFRTPPKDSTGIPHELIVERLTVLGCGRKYPVKEPFVELLKGSLHTFLNAFTYP
NP_175386 DRTCYPVASTNKKDFYNLVDVYLDAVFFPKCVDDVHTFQQEGWHYELNDPSEDISTKVFGIVFRTPPKDSTGIPHELIVERLTVLGCGRKYPVKEPFVELLKGSLHTFLNAFTYP

BAB02957  DRTCYPVASTNKKDFYNLVDVYLDAVFFPKCVDDVHTFQQEGWHYELNDPSEDISTKVFGIVFRTPPKDSTGIPHELIVERLTVLGCGRKYPVKEPFVELLKGSLHTFLNAFTYP
AAG13049  DRTCYPVASTNKKDFYNLVDVYLDAVFFPKCVDDVHTFQQEGWHYELNDPSEDISTKVFGIVFRTPPKDSTGIPHELIVERLTVLGCGRKYPVKEPFVELLKGSLHTFLNAFTYP
AAL67002  DRTCYPVASTNKKDFYNLVDVYLDAVFFPKCVDDVHTFQQEGWHYELNDPSEDISTKVFGIVFRTPPKDSTGIPHELIVERLTVLGCGRKYPVKEPFVELLKGSLHTFLNAFTYP
NP_175386 DRTCYPVASTNKKDFYNLVDVYLDAVFFPKCVDDVHTFQQEGWHYELNDPSEDISTKVFGIVFRTPPKDSTGIPHELIVERLTVLGCGRKYPVKEPFVELLKGSLHTFLNAFTYP

BAB02957  ARATEEELRLKQETPDPPDALKCVPSLNLSDIPKEPIYVPTEVGDINGVKVLRNDLFTNNILYTEVVFDMGSVKHELLQLIPLFCQSLLEMGTQDLTFVQL
AAG13049  ARATEEELRLKQETPDPPDALKCVPSLNLSDIPKEPIYVPTEVGDINGVKVLRNDLFTNNILYTEVVFDMGSVKHELLQLIPLFCQSLLEMGTQDLTFVQL
AAL67002  ARATEEELRLKQETPDPPDALKCVPSLNLSDIPKEPIYVPTEVGDINGVKVLRNDLFTNNILYTEVVFDMGSVKHELLQLIPLFCQSLLEMGTQDLTFVQL
NP_175386 ARATEEELRLKQETPDPPDALKCVPSLNLSDIPKEPIYVPTEVGDINGVKVLRNDLFTNNILYTEVVFDMGSVKHELLQLIPLFCQSLLEMGTQDLTFVQL

Figure 4
A

P1: Dye-Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys
P2: Dye-Leu-Arg-Arg-Ala-Ser-Leu-Gly

B

| Lane | 1 | 2 |
|------|---|---|
| P1   | + | + |
| MiQ2 | - | + |

C

| Lane | 1 | 2 |
|------|---|---|
| P2   | + | + |
| MiQ2 | - | + |

Figure 5
**Figure 6**

### A

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|------|---|---|---|---|---|---|---|
| $N_{v}pF_{i}\beta$ | + | + | + | + | + | + | |
| MPP | + | + | + | + | + | + | + |
| MiQ2 | + | + | + | + | + | + | + |
| inhibitor | − | − | PMSF | NEM | o-ph | cpi | apyrase |

### B

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|------|---|---|---|---|---|---|---|
| P1 | + | + | + | + | + | + | + |
| MiQ2 | − | − | PMSF | NEM | o-ph | cpi | apyrase |

**inhibitor**

**cathode** −

**anode** +
| Lane | 1 | 2 |
|------|---|---|
| N\(_{\alpha}\)pF\(_{\beta}(2-54)\)hsl | + | + |
| MiQ2 | − | + |

**Figure 7**

The image shows a gel electrophoresis with bands at 6.5 and 3.4 kDa.
Isolation and identification of a novel mitochondrial metalloprotease (PreP) that degrades targeting presequences in plants
Annelie Ståhl, Per Moberg, Jimmy Ytterberg, Oleg Panfilov, Helena Brockenhuis von Löwenhielm, Fredrik Nilsson and Elzbieta Glaser

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