Recognition and processing of a nuclear-encoded polypeptide precursor by mitochondrial processing peptidase

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The nuclear-encoded protein RPS14 (ribosomal protein S14) of rice mitochondria is synthesized in the cytosol as a polypeptide precursor consisting of a large N-terminal domain comprising preSDHB (succinate dehydrogenase B precursor) and the C-terminal RPS14. After the preSDHB–RPS14 polypeptide is transported into the mitochondrial matrix, the protein is processed into three peptides: the N-terminal prepeptide, the SDHB domain and the C-terminal mature RPS14. Here we report that the general MPP (mitochondrial processing peptidase) plays an essential role in processing of the polypeptide. Purified yeast MPP cleaved both the N-terminal presequence and the connector region between SDHB and RPS14. Moreover, the connector region was processed more rapidly than the presequence. When the site of cleavage between SDHB and RPS14 was determined, it was located in an

MPP processing motif that has also been shown to be present in the N-terminal presequence. Mutational analyses around the cleavage site in the connector region suggested that MPP interacts with multiple sites in the region, possibly in a similar manner to the interaction with the N-terminal presequence. In addition, MPP preferentially recognized the unfolded structure of preSDHB–RPS14. In mitochondria, MPP may recognize the stretched polypeptide during passage of the precursor through the translocational apparatus in the inner membrane, and cleave the connecting region between the SDHB and RPS14 domains even before processing of the presequence.

Key words: mitochondria, polypeptide, processing, protease, protein transport.

INTRODUCTION

Most mitochondrial proteins are encoded by the nucleus and synthesized by cytoplasmic ribosomes as precursor proteins with N-terminal presequences that are required for targeting to the mitochondria [1,2]. The precursor proteins are unfolded and imported into the mitochondrial matrix across the double membrane through the protein translocation machinery, Tom (translocase of the outer mitochondrial membrane) and Tim (translocase of the inner mitochondrial membrane) [3–6]. Finally, presequences are cleaved by a matrix-located metalloendoprotease, MPP (mitochondrial processing peptidase) [7–9]. Proteolytic processing is involved in the maturation of the mitochondrial proteins, and therefore the protease action is likely to be highly regulated and specific.

MPP is a heterodimer consisting of two structurally related subunits, α - and β -MPP. β -MPP is the catalytic subunit and contains a zinc ion at the active site [10,11], while α -MPP may be a regulatory subunit, providing substrate recognition [12,13]. MPP recognizes multiple sites in the presequence and generally cleaves at a single site. The general motif in the presequence for efficient processing is represented as the sequence (R/K) X_n RX↓ $\Phi\Psi\Psi$ (Φ and Ψ indicate hydrophobic and hydrophilic residues respectively, and the arrow indicates the cleavage point). However, cleavage by MPP is not completely lost in the presence of a defect in an element in the motif [14–18]. In addition, a glycine-induced flexible conformation of the presequence is preferred for processing [19]. The crystal structures of recombinant yeast MPP and presequence peptides complexed with a cleavage-deficient mutant of

MPP have been determined [20]. These crystal structures have revealed that MPP binds the substrate peptide in an extended conformation within a large polar cavity. Thus MPP recognizes the flexible presequence on the multiple binding sites arranged inside the molecular cavity [20,21].

In principle, one mRNA of nuclear origin encodes one mitochondrial protein. Genomic studies, however, have reported that one mRNA transcribed from the nuclear genome can encode two mitochondrial proteins in tandem [22–28]. The mRNA is translated as a polypeptide precursor, and the polypeptide carries multiple proteins with a single presequence at the N-terminus. In *Neurospora crassa*, two mitochondrial enzymes, AGK (*N*-acetylglutamate kinase) and AGPR (*N*-acetyl- γ -glutamyl-phosphate reductase), are encoded by a single nuclear gene and synthesized as a cytosolic polypeptide precursor [24]. After the polypeptide is imported into mitochondria, both the N-terminal targeting presequence and the connecting region between the AGK and AGPR domains are processed by a metal-dependent peptidase [24]. Although maturation of the polypeptide by MPP purified from *Neurospora* has been observed *in vitro*, how MPP reacts with the polypeptide and the determinant for cleavage at the central region of the protein is unknown.

Among such nuclear-encoded mitochondrial polypeptide precursors, the gene structure and transcription of the genes encoding RPS14 (ribosomal protein S14) and SDHB (succinate dehydrogenase subunit B) is of particular interest, and has been well characterized in plants. Although the functional gene encoding RPS14 is located in the mitochondrial genomes of the broad bean, *Oenothera* and pea [29–31], the *Arabidopsis thaliana*, maize and

Abbreviations used: AGK, *N*-acetylglutamate kinase; AGPR, *N*-acetyl- γ -glutamyl-phosphate reductase; GST, glutathione S-transferase; MPP, mitochondrial processing peptidase; RPS14, ribosomal protein S14; SDHB, succinate dehydrogenase subunit B; Tim, translocase of the inner mitochondrial membrane; Tom, translocase of the outer mitochondrial membrane.

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Figure 1 Gene and protein structures of rice SDHB and RPS14, and of the polyprotein precursor

(A) Schematic representation of the rice nuclear *rps14* and *sdhB* genes, and the translated proteins. Grey boxes indicate the SDHB region, and black boxes show the RPS14 region. White boxes represent the presequences and connector regions. The destinations of the mature proteins after processing of the preproteins are indicated. (B) Domain structure of preSDHB-RPS14. Grey and black boxes indicate SDHB and RPS14 domains respectively. The amino acid sequence of the connector region between the SDHB and RPS14 domains is shown. The calculated molecular masses of the respective regions are indicated.

rice genes are encoded in the nucleus [26–28]. Furthermore, it has been shown that rice and maize *rps14* has become integrated between two exons of a *SDHB* gene (Figure 1). Alternative splicing generates two transcripts from the *SDHB-rps14* locus: one encoding a chimaeric protein precursor formed by the fusion of C-terminally truncated SDHB to RPS14 [the SDHB-RPS14 precursor (preSDHB-RPS14)], and the other encoding a full-length SDHB precursor (Figure 1) [27,28]. The former chimaeric precursor is processed into the SDHB domain and functional RPS14 in mitochondria [28,32]. The latter may be converted into mature SDHB after removal of the N-terminal presequence by MPP. Although processing of preSDHB-RPS14 is required for the generation of functional RPS14 in rice and maize, this processing has not yet been well characterized.

In the present study, we examined whether MPP specifically recognizes and processes the polyprotein precursor *in vitro*, and what the determinant is for processing. We found that (1) MPP cleaves preSDHB-RPS14 not only at the N-terminal presequence, but also at the connector region between the SDHB and RPS14 domains; (2) multiple sites in the connector region are required for cleavage; and (3) the extended structure of the polyprotein precursor is involved in processing. We discuss possible mechanisms for polyprotein recognition and processing by MPP.

EXPERIMENTAL

Yeast culture conditions and preparation of mitochondria

Saccharomyces cerevisiae strain INV sc1 (Invitrogen) was grown on medium containing 3 g of yeast extract, 0.5 g of glucose, 0.5 g of CaCl₂, 0.5 g of NaCl, 0.6 g of MgCl₂, 1 g of KH₂PO₄, 1 g of NH₄Cl, 5 g of casamino acids, 1.3 mg of amino acids complete, 20 mg of uracil and 22 ml of 90% (v/v) acetic acid per litre. The

final pH was adjusted to 5.6 with NaOH. Cells were grown to $D_{600} = 1$ at 30 °C, washed with distilled water, suspended in 50 ml of 0.1 M Tris/SO₄ (pH 9.4) containing 10 mM dithiothreitol, and incubated for 30 min at 30 °C. The cells were harvested by centrifugation for 5 min at 2000 g, and suspended in 20 mM potassium phosphate buffer (pH 7.4) containing 1.2 M sorbitol (sorbitol buffer). Zymolyase was added to the cell suspension, and the spheroplasts were harvested by centrifugation for 5 min at 6000 g at 4 °C. Harvested cells were suspended in 20 mM Hepes/KOH (pH 7.4) containing 0.6 M mannitol, 1 mM PMSF and 5 mM EDTA (breaking buffer), and the cells were homogenized. The homogenate was harvested after centrifugation for 5 min at 1000 g, and the pellet was homogenized again. The post-nuclear supernatant was centrifuged at 12000 g. The pellet was washed once with 10 mM Mops/KOH (pH 8.0) containing 250 mM sucrose and 5 mM EDTA (SEM buffer). The mitochondrial pellet was finally suspended in SEM buffer.

Mutations and synthesis of radiolabelled proteins

The cDNA encoding the rice SDHB-RPS14 precursor was digested with *Eco*RI and inserted into the pGEM-4Z vector. Amino acid substitutions were made by replacing codons with the appropriate oligonucleotides using a QuickChange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing. Radiolabelled proteins were synthesized using a TNT SP6 coupled reticulocyte lysate system (Promega) and labelled with [³⁵S]methionine for 90 min at 30 °C.

In vitro import into mitochondria

Yeast mitochondria (100 µg) were incubated for 5 min at 30 °C with 1 µl of 0.2 M NADH (dissolved in SEM buffer) and 90 µl of import mixture [10 mM Mops/KOH, pH 7.2, containing 250 mM sucrose, 1% (w/v) BSA, 80 mM KCl, 5 mM MgCl₂, 2 mM potassium phosphate, 5 mM dithiothreitol, 20 mM ATP (pH 7.0), 20 mM phosphocreatine and 200 µg of creatine kinase]. Radiolabelled protein precursors were incubated with the import-competent mitochondria for various times at 30 °C, and the mitochondria mixture was then incubated with 1 µg of valinomycin and 10 µg of proteinase K for 30 min on ice. The mitochondria were then treated with 1 mM PMSF and washed once with SEM buffer. Imported protein was detected by SDS/PAGE, followed by imaging and image analysis (Fuji Film; BAS 1500).

In vitro processing of the polyprotein by MPP

Recombinant yeast MPP was expressed in *Escherichia coli* BL21 (DE3) strain, and purified using a nickel-chelating Sepharose column (Amersham Biosciences) as described previously [10]. Radiolabelled proteins were incubated with purified MPP in 10 mM Hepes/KOH (pH 7.4) containing 0.1% (v/v) Tween 20 and 0.5 mM MnCl₂ at 30 °C. The reactions were stopped by the addition of SDS to a final concentration of 2% (w/v). Processed products were analysed by SDS/PAGE using an imaging analyser or by protein staining with Coomassie Brilliant Blue R250.

Expression and purification of tagged SDHB-RPS14 polyprotein precursors

A hexahistidine tag was introduced at the C-terminus of the SDHB-RPS14 polyprotein precursor using PCR, and the product was inserted into the expression vector pET-23d. The expression vector was transformed into *E. coli* BL21 (DE3), and the cells were cultured in Luria-Bertani medium containing 0.2 mM isopropyl β-D-thiogalactoside for 20 h at 25 °C. The SDHB-RPS14

polypeptide precursor was purified using a nickel-chelating Sepharose column [10]. To fuse the SDHB-RPS14 polypeptide precursor to GST (glutathione S-transferase), a cDNA encoding the polypeptide tagged with a C-terminal hexahistidine was inserted into the vector pGEX-2T (Amersham Biosciences). The GST fusion polypeptide, GST-SDHB-RPS14, was expressed in *E. coli* BL21 (DE3) as described above. The cells producing the GST-SDHB-RPS14 polypeptide were harvested by centrifugation at 1000 *g* for 10 min, and suspended in PBS. Proteins were extracted by sonication. The suspension was centrifuged at 10000 *g* for 20 min. The resultant supernatant was loaded on a glutathione-Sepharose 4B column (Amersham Biosciences; GStrap) equilibrated with PBS. The column was washed with PBS. The GST fusion proteins were eluted with 50 mM Tris/HCl (pH 8.0) containing 15 mM glutathione, 15 % (v/v) glycerol and 0.01 % (v/v) Tween 20. The eluate was diluted 10-fold with 20 mM Hepes/KOH (pH 7.4) containing 30 % glycerol and 0.01 % Tween 20. GST-SDHB-RPS14 was purified further using a nickel-chelating Sepharose column and used for processing analyses. Unfolded GST-SDHB-RPS14 was prepared as described previously [21] and used to determine the site of cleavage by MPP.

N-terminal amino acid sequencing

Proteins were separated by SDS/PAGE and transferred to an Immobilon-P Transfer Membrane (Millipore) by electroblotting in 48 mM Tris, 39 mM glycine, 0.0375 % (w/v) SDS and 16 % methanol. Proteins on the membrane were stained with Coomassie Brilliant Blue G250. Protein bands of interest were cut out and the N-terminal amino acid sequences were determined using an automated protein sequencer (Model 491A; Applied Biosystems).

RESULTS

The SDHB-RPS14 polypeptide precursor is imported into isolated yeast mitochondria and processed

To investigate whether rice preSDHB-RPS14 is imported into isolated mitochondria and processed into the mature polypeptides, we performed a mitochondrial import assay using radiolabelled preSDHB-RPS14. After incubation of the polypeptide with yeast mitochondria, the precursor was converted into polypeptides of 27 kDa and 12 kDa (Figure 2A, lane 2), which were resistant to added proteinase K, indicating that the protein was processed by a peptidase(s) in the mitochondria. Protein transport depended on the membrane potential across the inner membrane, as processing was abolished by the addition of valinomycin, a potassium ionophore, which diminishes the membrane potential (Figure 2A, lane 3), indicating that the polypeptide was imported into mitochondria through the general protein translocation pathway. After pre-incubation of mitochondria with the metal chelators 1,10-phenanthroline and EDTA, processing was inhibited, and the polypeptide precursor accumulated in the mitochondria even with proteinase K treatment (Figure 2A, lane 4). Polypeptide processing in the mitochondria was observed early in protein import (Figure 2B). These results suggested that a metal-dependent protease(s), possibly MPP, cleaves preSDHB-RPS14 during or after import into the mitochondrial matrix.

MPP cleaves the polypeptide efficiently between the SDHB and RPS14 domains

To investigate the direct participation of MPP in the processing of preSDHB-RPS14, the radiolabelled precursor was incubated with various amounts of purified recombinant yeast MPP. As shown in Figure 3(A), preSDHB-RPS14 was processed into four frag-

Figure 2 Import of preSDHB-RPS14 into isolated yeast mitochondria

(A) Polypeptide import into isolated mitochondria. The import assay was performed as described in the Experimental section. Isolated yeast mitochondria were pre-incubated for 5 min at 30 °C in import buffer without (lanes 2 and 4) or with (lane 3) valinomycin, which inhibits protein import into mitochondria. 1,10-Phenanthroline (o-Phe) and EDTA (lane 4) were used as inhibitors of metal-dependent proteases. Radiolabelled polypeptide precursor was added to the import mixture, and the incubation was continued for 15 min at 30 °C. The mitochondria were then re-isolated by centrifugation, and the mitochondrial pellet was treated with proteinase K at 0 °C for 30 min. The samples were subjected to SDS/PAGE and analysed using an imaging analyser. Lane 1 contains radiolabelled polypeptide precursor without mitochondria. The arrowheads indicate the polypeptide precursor and the processing products in the mitochondria. (B) Kinetic analysis of polypeptide import. Radiolabelled preSDHB-RPS14 was incubated with isolated yeast mitochondria in import mixture at 30 °C for the times indicated, and the imported proteins were analysed as described above.

ments by MPP. These fragments were identified as SDHB-RPS14 (34 kDa), preSDHB (27 kDa), SDHB (22 kDa) and RPS14 (12 kDa) from the molecular masses predicted using SDS/PAGE. The major processing products were preSDHB and RPS14 upon incubation with a small amount of MPP. However, one of the major processing products resulting from incubation with high concentrations of MPP was SDHB rather than preSDHB, indicating that a large amount of MPP was needed to generate the 22 kDa SDHB domain. Therefore it was likely that MPP cleaved a site in the connector region between SDHB and RPS14 more efficiently than the N-terminal presequence. To confirm this, we analysed the kinetics of the processing reaction. Two polypeptide products, preSDHB and RPS14, were generated earlier than SDHB-RPS14 and SDHB during processing (Figure 3B). Thus MPP cleaved the SDHB-RPS14 polypeptide precursor not only at the presequence-SDHB junction, but also in the SDHB-RPS14 connector region, and this latter site was processed more efficiently.

Identification of the site of cleavage of the polypeptide precursor

MPP generally cleaves the N-terminal presequence of short peptides that are no longer than 50 amino acids. Therefore it is notable that MPP efficiently cleaved the middle region of the target protein in this case. To elucidate the molecular recognition and processing of the polypeptide by MPP, we first tried to determine the site of cleavage between the SDHB and RPS14 domains. Recombinant GST-SDHB-RPS14 polypeptide, in which GST was fused to the N-terminus of the SDHB domain, was used to determine the processing site, because a large amount of substrate was needed for protein sequencing. After purification of GST-SDHB-RPS14, the unfolded polypeptide in urea was diluted rapidly into a large volume of the processing buffer containing MPP. After incubation, cleavage products of 53 kDa and 12 kDa were detected (Figure 4A). Edman sequencing of the two polypeptides revealed that the N-terminal amino acid sequence of the 53 kDa protein corresponded to that of GST, and that the seven N-terminal amino acid residues of the 12 kDa protein were Tyr-His-Gly-Val-Ser-Glu-Lys, which is the same as the amino acid sequence from Tyr-248

Figure 3 Processing of preSDHB-RPS14 by MPP

(A) Dose-dependent processing of preSDHB-RPS14 by MPP. Radiolabelled preSDHB-RPS14 was incubated with purified yeast MPP (0, 25, 50, 100 and 200 ng of MPP in lanes 1–5 respectively) at 30 °C for 30 min. SDS/PAGE was performed on the cleaved polypeptides and they were visualized using an imaging analyser. The arrows indicate the precursor protein and the processing products with the calculated molecular masses. (B) Kinetic analysis of polyprotein processing. Radiolabelled preSDHB-RPS14 was incubated with purified yeast MPP (50 ng) in processing assay buffer at 30 °C for the times indicated. The rate of cleavage of each polypeptide region is shown.

to Lys-254 of preSDHB-RPS14 (Figure 4B), indicating that MPP cleaves the peptide bond between Gly-247 and Tyr-248. The RPS14 domain is considered to start at Ser-252 of the precursor protein when the similarity between the amino acid sequences of SDHB and RPS14 proteins from several species is taken into account [28]. Thus MPP definitely cleaves in the connector region between SDHB and RPS14.

Elements required for processing of the polyprotein by MPP

MPP recognizes the basic N-terminal presequence of preSDHB-RPS14 and preferentially cleaves peptide bonds between –2 arginine and +1 hydrophobic residues. The connector region of preSDHB-RPS14 is highly basic, and the cleavage site, which was determined in the previous experiment to be Arg-Gly↓Tyr-His-Gly, contains arginine and hydrophobic residues at the –2 and +1 positions respectively. It is of a great interest to determine if MPP cleaves the middle region of the protein molecule by the same recognition mechanism as is involved in cleavage of the N-terminal presequence. Therefore single or multiple substitutions were introduced into preSDHB-RPS14, focusing on the distal basic residues at positions –23, –14, –12 and –6, the arginine residue at the –2 position, the tyrosine residue at the +1 position, the histidine residue at the +2 position and the glycine residue at the +3 position (Table 1).

Figure 4 Determination of the site of cleavage between SDHB and RPS14 by MPP

(A) Cleavage of the GST fusion polyprotein by MPP. PreSDHB-RPS14 fused to GST at the N-terminus and with a hexahistidine tag at the C-terminus (GST-SDHB-RPS14) was mostly produced as inclusion bodies in *E. coli* extract. The polyprotein was then denatured and purified as described in the Experimental section. The unfolded GST fusion polyprotein was incubated with purified yeast MPP in processing assay buffer at 30 °C for 90 min. The processed products were separated by SDS/PAGE and stained with Coomassie Brilliant Blue R-250. Arrows indicate the processing products (GST-SDHB and RPS14). The N-terminal amino acid sequences of the two bands were determined. (B) Site of cleavage between the SDHB and RPS14 regions of the polyprotein. The N-terminal amino acid sequence of GST-SDHB-RPS14 is shown with the processing site that was determined by direct protein sequencing. Actual protein sequence is denoted by an underline.

Table 1 Mutation sites in the connector region of preSDHB-RPS14

Substituted amino acids are in bold and underlined, and the MPP cleavage site (between Gly-247 and Tyr-248) is indicated by the arrow.

Mutant protein	Amino acid sequence
Wild type	...ANRLPLLGLTIKPKPNMFMHIQARG↓YHGV ²⁵¹ ...
KK-AA	...ANRLPLLGLTI <u>IA</u> PNMFMHIQARG↓YHGV ²⁵¹ ...
RKK-AAA	...AN <u>AL</u> PLLGLTI <u>IA</u> PNMFMHIQARG↓YHGV ²⁵¹ ...
KKH-AAA	...ANRLPLLGLTI <u>IA</u> PNMFM <u>AI</u> QARG↓YHGV ²⁵¹ ...
RKKH-4A	...AN <u>AL</u> PLLGLTI <u>IA</u> PNMFM <u>AI</u> QARG↓YHGV ²⁵¹ ...
A-2	...ANRLPLLGLTIKPKPNMFMHIQ <u>AG</u> ↓YHGV ²⁵¹ ...
K-2	...ANRLPLLGLTIKPKPNMFMHIQ <u>AK</u> ↓YHGV ²⁵¹ ...
G-2	...ANRLPLLGLTIKPKPNMFMHIQ <u>AGG</u> ↓YHGV ²⁵¹ ...
A+1	...ANRLPLLGLTIKPKPNMFMHIQARG↓ <u>A</u> HGV ²⁵¹ ...
F+1	...ANRLPLLGLTIKPKPNMFMHIQARG↓ <u>F</u> HGV ²⁵¹ ...
L+1	...ANRLPLLGLTIKPKPNMFMHIQARG↓ <u>L</u> HGV ²⁵¹ ...
G+1	...ANRLPLLGLTIKPKPNMFMHIQARG↓ <u>G</u> HGV ²⁵¹ ...
A+2	...ANRLPLLGLTIKPKPNMFMHIQARG↓Y <u>AG</u> V ²⁵¹ ...
S+2	...ANRLPLLGLTIKPKPNMFMHIQARG↓Y <u>SG</u> V ²⁵¹ ...
T+2	...ANRLPLLGLTIKPKPNMFMHIQARG↓Y <u>TG</u> V ²⁵¹ ...
S+3	...ANRLPLLGLTIKPKPNMFMHIQARG↓YH <u>S</u> V ²⁵¹ ...
T+3	...ANRLPLLGLTIKPKPNMFMHIQARG↓YH <u>T</u> V ²⁵¹ ...
HG-ST	...ANRLPLLGLTIKPKPNMFMHIQARG↓Y <u>ST</u> V ²⁵¹ ...

All or some of the N-terminal four basic residues at positions –23, –14, –12, and –6 from the cleavage site, which potentially act as the distal basic elements involved in recognition by MPP,

Figure 5 Effects of mutations in the connector region of the polyprotein on cleavage between the SDHB and RPS14 domains by MPP

Radiolabelled polyprotein precursor was incubated with purified yeast MPP (50 ng) for 4 min at 30 °C. Processed products were subjected to SDS/PAGE and visualized using an imaging analyser. Processing efficiency was determined by quantifying the band density of the cleaved polyprotein precursor. The graphs show the processing efficiency of various polyproteins mutated at positions on the N-terminal side (A) and the C-terminal side (B) of the cleavage site, taking the processing efficiency of MPP for wild-type (WT) polyprotein precursor as 100%. The processing efficiencies were calculated from three independent experiments. The amino acid sequences of the various mutants are given in Table 1.

were replaced with alanine residues. The rates of cleavage of all polyprotein mutants were substantially lower than that of the wild-type protein (Figure 5A). Furthermore, the efficiency of processing was dependent on basicity in the connector region, indicating that positively charged amino acids in this region are involved in the enhancement of processing by MPP.

As shown in Figure 5(A), mutation of the arginine residue at the -2 position reduced the processing rate. The cleavage rate of the A-2 mutant polyprotein (i.e. with Ala at the -2 position) was decreased to about half that of the wild-type protein, and protein processing of G-2 was undetectable. The K-2 protein was cleaved more efficiently than A-2, but its rate of cleavage was lower than that of the wild-type protein, suggesting that the guanido group is required for effective processing at the -2 site. Thus, even for cleavage in the connector region of the preSDHB-RPS14 polyprotein, the arginine residue at the -2 position is involved in effective processing.

We examined whether a hydrophobic amino acid residue at the +1 site also allowed effective processing of the polyprotein by MPP. When the tyrosine residue at the +1 site was replaced with alanine, phenylalanine or leucine, the processing efficiency was half that of the wild-type protein (Figure 5B). A glycine residue at this position resulted in an undetectable level of processing. Thus a hydrophobic amino acid residue at the +1 position is also

an important determinant in the MPP cutting site for producing RPS14 from preSDHB-RPS14.

Finally, we analysed the effects of amino acid residues downstream from the cleavage site on processing. It has been demonstrated previously that replacement of the residues at the +2 and +3 sites of mouse malate dehydrogenase preprotein results in a significant decrease in processing, and that MPP prefers serine and threonine residues at these sites [16]. In the SDHB-RPS14 polyprotein, the +2 and +3 sites contain histidine and glycine respectively. These residues are hydrophilic, but are not serine and threonine. To examine the roles of the hydrophilic residues, we replaced the amino acids at the +2 and +3 sites with serine, threonine or alanine residues, and analysed the cleavage efficiency. These mutated proteins had a reduced cleavage rate, and there was no preference for serine and threonine residues (Figure 5B). We had expected that the double-site mutant HG-ST protein (in which His and Gly at positions +2 and +3 are replaced by Ser and The respectively) would be cleaved more efficiently than the wild-type protein, but the rate of processing of this protein was approximately half that of the wild-type protein. Thus the importance of a hydroxy group at the +2 and +3 positions for the processing of the SDHB-RPS14 polyprotein is not clear.

An unfolded structure is essential for processing of the preSDHB-RPS14 polyprotein by MPP

The N-terminal presequence of mitochondrial precursor proteins has a random conformation in aqueous solution, and such an extended and flexible structure is required for processing by MPP [19]. In the processing of preSDHB-RPS14, the cleavage point is located in the centre of a large molecule. Therefore we questioned if MPP can interact with a polyprotein substrate with a highly ordered structure, or if an unfolded structure is required between the two domains for cleavage. We analysed the relationship of trypsin sensitivity to MPP processing for the polyprotein by using two types of protein, SDHB-RPS14 and GST-SDHB-RPS14 precursors, which were purified under non-denaturing conditions. With the GST fusion polyprotein, the 35 kDa products remained even after stringent trypsin treatment, indicating that the 35 kDa domain is highly resistant to trypsin digestion (Figure 6A). As the trypsin fragments reacted with the anti-(His tag) antibody, these polypeptides lost the N-terminal GST region instead of the C-terminal tag portion. These results suggested that the GST-SDHB-RPS14 fusion polyprotein has a large trypsin-insensitive central-core domain consisting of SDHB-RPS14. On the other hand, non-fused polyprotein precursor was mostly degraded by low amounts of trypsin. Intermediate fragments were barely observed by detection with anti-(His tag) antibody or silver staining (results not shown), indicating that preSDHB-RPS14 was highly sensitive to trypsin. Therefore it appeared that the whole structure of preSDHB-RPS14 is not rigid, but is unfolded and extended. In processing by MPP, preSDHB-RPS14 was cleaved efficiently by MPP, while GST-SDHB-RPS14 was not cleaved at all (Figure 6B). However, the GST fusion polyprotein denatured in urea was cleaved by MPP, although the amount of MPP used was different (Figure 4A). From these observations, MPP seems to interact preferentially with the polyprotein in an unfolded or stretched form, and cleaves it at a specific site in the connector region.

DISCUSSION

Previously, it was believed that MPP interacts with the short stretch region of N-terminal mitochondrial preproteins and hardly at all with the central region in the mature portion. However, in the present study, we examined the processing of a rice mitochondrial

Figure 6 Unfolding requirement for processing of preSDHB-RPS14 by MPP

(A) Trypsin sensitivity of purified polyproteins. Purified protein (100 ng) was incubated with various concentrations of trypsin (0, 0.01, 0.1, 0.5, 1, 2.5, 5, 10 or 50 ng of trypsin in lanes 1–9 respectively) for 15 min at 30 °C. The polyprotein precursors and the degraded C-terminal polypeptides were analysed by immunoblotting with anti-hexahistidine antibodies and peroxidase-coupled anti-mouse secondary antibodies. (B) Processing of purified polyproteins by MPP. SDHB-RPS14 and GST-SDHB-RPS14 polyprotein precursor were expressed in *E. coli*, and purified from the soluble fraction. Purified proteins (100 ng) were incubated with various concentrations of MPP (0, 1, 10 or 100 ng of MPP in lanes 1–4 respectively) in processing assay buffer for 30 min at 30 °C. All samples were subjected to SDS/PAGE and gels were analysed by silver staining.

polyprotein, preSDHB-RPS14, by MPP, as well as the structural features involved in processing of the polyprotein. We propose that MPP is not only a signal peptidase, but also plays an important role in mitochondrial polyprotein processing.

We show that MPP cleaves the connector region of the SDHB-RPS14 polyprotein, and that several structural elements in the connector region, i.e. multiple basic sites, an arginine at the –2 position and a hydrophobic residue at the +1 position, are required for effective processing. We show that the unfolded polyprotein is cleaved efficiently. Crystal structures of a yeast MPP–substrate peptide complex revealed that MPP incorporates the presequence into the internal molecular cavity, possibly through a narrow cleft between the subunits, and cleaves it in the hydrophilic environment [20]. Since the cleft leading to the cavity of MPP is narrow, it seems likely that the folded polypeptide would have difficulty in entering the cavity. As we demonstrated that an unfolded state is essential for polyprotein processing, the connector region may enter the narrow cleft of the MPP cavity in an extended conformation. We have proposed previously a possible model by which MPP identifies the site in presequences for processing [21]. During polyprotein processing, MPP may search progressively for a sequence motif in the substrate starting at the N-terminus and identify appropriate sites that fit the processing motif $RX\downarrow\Phi\Psi\Psi$ (where Φ and Ψ indicate hydrophobic and hydrophilic residues respectively).

Protein transport into mitochondria is a dynamic process. The participation of membrane proteins such as Tom and Tim and molecular chaperones such as Hsp70s is required for the translocation system [3–6]. Preproteins are in an extended conformation during transport into mitochondria through the Tom and Tim complexes, and mitochondrial chaperones keep the transit proteins unfolded and prevent misfolding and protein aggregation. Since the processing motif in unfolded and extended polypeptides is involved in interaction with and cleavage by MPP, the best

opportunity for MPP to recognize and cleave the unfolded polypeptide probably occurs when the proteins are being imported into the matrix or have just been imported. Indeed, transport intermediate preproteins are capable of being processed by MPP [33], and the cleavage between the two domains of the SDHB-RPS14 polyprotein was detected at an early stage of import into mitochondria in the present study. MPP possibly interacts with the extended polyprotein during transit through the Tim channel.

Why are some mitochondrial proteins encoded by a single gene and linked to each other as polyprotein precursors? What is the biological significance of encoding mitochondrial proteins in the form of a polyprotein? In *N. crassa*, AGK and AGPR are also synthesized as a mitochondrial polyprotein precursor, preAGK-AGPR [24]. AGK and AGPR are the second and third enzymes respectively in the eight-step biosynthesis of arginine from glutamate. These two early steps in arginine biosynthesis are thus regulated by the transcription of a single gene in *N. crassa*. Although the concept that MPP regulates enzyme activities by proteolytic processing is very interesting, this possibility seems unlikely, as arginine biosynthesis activity has been demonstrated even in the polyprotein form [24]. Therefore the biological advantage of the AGK-APGR polyprotein may be just economical in terms of gene structure, transcription/translation and protein transport.

SDHB and RPS14, on the other hand, show no direct enzymic relationship. A previous report indicated that, not only in rice but also in maize, mitochondrial RPS14 is synthesized as a polyprotein that is fused to preSDHB and processed to the short form of SDHB and mature RPS14 after import into mitochondria. The short form of SDHB may be degraded, because it is undetectable in maize mitochondria by antisera against SDHB [32]. Moreover, full-length SDHB is translated from alternatively spliced transcripts in rice and maize [28,32]. Therefore the biological significance of the preSDHB-RPS14 polyprotein is regarded as being the generation of a mitochondrial targeting function for RPS14 [28,32]. This interpretation may be of importance when we consider presequence generation during the evolution of mitochondria. The endosymbiont hypothesis has generally been accepted as explaining the origin of mitochondria, and most of the genetic information in the endosymbiont was transferred to the nuclear genome of the host cell. Before, during or after gene transfer, presequences were added to the proteins to allow import into mitochondria. By this time, the protein transport and processing system should have been constructed. A number of genes may have been recombined and fused to each other during the evolutionary process. Therefore the gene structure of the preSDHB-RPS14 polyprotein might be an intermediate step in presequence generation. The molecular recognition and relationship between construction of the presequence and the MPP processing system is one of the most interesting and important events during mitochondrial evolution. When we are in the post-genome stage, comparison and detailed analyses of the gene structures of mitochondrial proteins will be required in order to understand not only mitochondrial functions but also mitochondrial evolution.

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