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Substrate Recognition by Mitochondrial Processing Peptidase toward the Malate Dehydrogenase Precursor

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Mitochondrial processing peptidase (MPP) cleaves the extension peptides of precursor proteins newly imported into the mitochondria. Using synthetic oligopeptides modeled on the extension peptide of malate dehydrogenase, the critical elements of the substrate for the processing of MPP were determined [Niidome, T., Kitada, S., Shimokata, K., Ogishima, T., and Ito, A. (1994) *J. Biol. Chem.* 269, 24719-24722; Ogishima, T., Niidome, T., Shimokata, K., Kitada, S., and Ito, A. (1995) *ibid.* 270, 30322-30326]. In the present study, we constructed mutant precursors and compared the processing reaction with that of the peptide substrates to confirm the validity of use of peptide substrates. In both cases, the arginine residue presents at a proximal (−2) position relative to the processing site proved to be important for the processing. The distal arginine residue at position 7 was replaceable with alanine with no significant loss in cleavage efficiency if the precursor protein contained two consecutive arginine residues at a proximal position, although the arginine residue at a position 7 was indispensable in the model peptide. The proline residue, lying between the distal and proximal arginine residues, which is assumed to break a continuous α-helix region in the extension peptide, was needed for the processing. This peptidase has a preference for aromatic amino acids at the P_1' site.[2] These results were essentially the same as those obtained with model peptides except for the role of the distal arginine. We also found that amino acids at P_2' and P_3' sites had some effects on the processing. Thus we concluded that an effective combination of model peptides with precursor proteins is needed for the studies on MPP responsible substrate-recognition mechanisms.

Key words: extension peptide, malate dehydrogenase precursor, mitochondrial processing peptidase, peptide substrate, substrate recognition.

Most mitochondrial proteins are encoded in the nucleus, synthesized as precursor proteins with an extension peptide, and targeted for the organelle. After translocation into the mitochondria, the extension peptides are proteolytically cleaved off by a matrix-located metallopeptidase, mitochondrial processing peptidase (MPP) (*1-3*). This peptidase is a hetero dimer consisting of α and β subunits (*4-11*), of which the β subunit has a catalytic role (*12*). In some cases, MPP first produces intermediate proteins, then a mitochondrial intermediate peptidase (MIP) removes eight amino acids (octapeptide) from their amino terminus to produce mature proteins (*13*). MPP acts only on mitochondrial precursor proteins, not on mitochondrial mature proteins or secretory precursor proteins, and this indicates that MPP recognizes the extension peptides of mitochondrial precursor proteins. Since these extension peptides vary in length and sequence (*14-16*), but are rich in positively charged amino acids among the hydrophobic amino acids, MPP probably recognizes a higher order structure of the extension peptides.

Studies on the recognition and processing mechanisms (*17-21*) have revealed the importance of basic amino acids near the processing site together with the role of the amino acids in the middle portion of the extension peptide. Using synthetic peptides modeled on malate dehydrogenase (MDH), we kinetically analyzed the processing reaction in detail and revealed that an arginine residue in the N-terminal portion and arginine residue at position −2 relative to the cleavage site (distal and proximal positions from the cleavage site, respectively) are important for the processing (*22, 23*). We also obtained evidence that the presence of α-helix-breaking amino acids was needed between the distal and proximal arginine residues and that MPP had a preference for aromatic amino acids at P_1' position (*24*). The use of synthetic peptides as substrates has the advantages of allowing systematic substitution of amino acid residues and quantitative analysis of the processing reaction, and in this way we identified a common structure responsible for the recognition of the extension peptide by MPP. Nevertheless, it is necessary to confirm that peptides and precursor proteins are processed *via* the same mecha-

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[2] The nomenclature P_1', P_2', and P_3' follows the method of Schechter and Berger (*31*).

Abbreviations: HEPES, 2-[4-(hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MDH, malate dehydrogenase; MPP, mitochondrial processing peptidase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

nisms, since other mature portions could affect the structure of the extension peptide, and thereby modify the processing reaction.

We examined the validity of using model peptides by analyzing the processing of precursor proteins. The MDH precursor, pre-MDH, which undergoes two-step processing, was mutated at various sites including the proximal and distal arginine residues, glycine and proline residues between these arginine residues, and amino acids at P_1', P_2', and P_3' sites. We analyzed the processing of these mutants by MPP and confirmed that essentially the same processing mechanisms operate for the precursor and the peptide substrate.

EXPERIMENTAL PROCEDURES

Construction of Mutants—A plasmid carrying a cDNA of mouse MDH (*25*) was digested with *Eco*RI and *Sal*I and inserted into pBluescript SK. The mutated genes were produced by the polymerase chain reaction (PCR)-based overlap extension method. The mutagenesis primers contained mutations and newly introduced restriction sites. For example, to construct the 14A15A mutant DNA, pair primers of 5′-GCGGCTAGCGCGGCGCCGGCAGGACG-3′ and reversal primer for the N-terminal fragment, and 5′-CGCGCTA*GCCGCC*AGCTTCAGCACTTTCGG-3′ and universal primer for the C-terminal fragment (underlines are *Nhe*I sites and italic is alanine codon) were generated. The N-terminal fragment was digested with *Eco*RI and *Nhe*I, and the C-terminal fragment was digested with *Nhe*I and *Sal*I. Two fragments were ligated into pBluescript SK digested by *Eco*RI and *Sal*I. Mutations were confirmed by DNA sequencing. Restriction sites for other mutants are listed in Table I.

Synthesis of the Precursor Proteins—The wild-type and mutant cDNAs were digested with *Eco*RI and *Sal*I and ligated into SP-65. The mRNAs were transcribed by SP-6 RNA polymerase, and radio-labeled precursors were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine, as described (*26*).

Assay of Processing Activity—Radio-labeled precursors were incubated with purified MPP at 30°C for 10 min in reaction buffer consisting of 20 mM HEPES-KOH (pH 7.4), 0.1% Tween 20, and 0.5 mM $MnCl_2$. The reaction was stopped by adding 1 mM EDTA. The processing products were separated by SDS-PAGE and visualized by fluorography. The fluorograms were scanned using a Nikon Scantouch (Nikon, Tokyo), and their densities were quantified with the NIH image.

Materials—MPP was purified from bovine liver mitochondria by the method of Ou *et al.* (*6*). Restriction enzymes and DNA-modifying enzymes were from Boehringer Mannheim (Mannheim, Germany) or Nippon Gene (Toyama). SP-6 RNA polymerase was from TaKaRa Biomedicals (Otsu). [^{35}S]Methionine was the product of ICN Biomedicals (Costa Mesa, CA).

RESULTS AND DISCUSSION

Construction of Pre-MDH Mutants—Table I shows amino acid sequences of the extension peptides of mouse wild type and mutant MDHs together with newly introduced restriction sites (right column). Silent mutations were also carried out to create new restriction sites, if mutation sites did not coincide with the restriction sites. We designated mutants according to the substituted sites and resultant amino acids. For example, 14A means a mutant with Ala in place of Arg at position 14 (from the N-terminus). Mutations were directed to arginine residues at position 7, 14, and 15, proline and glycine residues at position 8 and 10, respectively, phenylalanine at position 17 (P_1' site), and the second and third amino acid residues in the C-terminal portion from the cleavage site (P_2' and P_3' sites, respectively).

Requirement of Arginine Residue—Most mitochondrial precursor proteins have arginine residues at position −2 or −3 and basic amino acids at a distal position relative to the cleavage site, respectively (*14-16*). Earlier studies using synthetic peptides suggested that these basic amino acids are significant for the processing (*22*). To determine

TABLE I. **Constructed mutants and restriction sites introduced into the oligonucleotides.** Substituted amino acids are underlined, and sites of cleavage by MPP and MIP are shown by arrowheads.

Mutant	Sequence (MPP ▼ MIP ▼)	Restriction site
Wild type	MLSALARPAGAALRRS-FSTSAQNN-mature	
14A	MLSALARPAGAAL<u>A</u>RS-FSTSAQNN-mature	*Hin*dIII
15A	MLSALARPAGAALR<u>A</u>S-FSTSAQNN-mature	*Hin*dIII
14A15A	MLSALARPAGAAL<u>AA</u>S-FSTSAQNN-mature	*Nhe*I
14A15K	MLSALARPAGAAL<u>AK</u>S-FSTSAQNN-mature	*Hin*dIII
7A	MLSALA<u>A</u>PAGAALRRS-FSTSAQNN-mature	*Pst*I
7A14A	MLSALA<u>A</u>PAGAAL<u>A</u>RS-FSTSAQNN-mature	*Pst*I, *Hin*dIII
7K14A	MLSALA<u>K</u>PAGAAL<u>A</u>RS-FSTSAQNN-mature	*Pst*I, *Hin*dIII
7K14A15K	MLSALA<u>K</u>PAGAAL<u>AK</u>S-FSTSAQNN-mature	*Pst*I, *Hin*dIII
8A10A14A	MLSALAR<u>A</u>A<u>A</u>AAL<u>A</u>RS-FSTSAQNN-mature	*Pst*I, *Hin*dIII
8A14A	MLSALAR<u>A</u>AGAAL<u>A</u>RS-FSTSAQNN-mature	*Pst*I, *Hin*dIII
10A14A	MLSALARP<u>A</u>AAAL<u>A</u>RS-FSTSAQNN-mature	*Eco*521, *Hin*dIII
14A17A	MLSALARPAGAAL<u>A</u>RS-<u>A</u>STSAQNN-mature	*Pvu*I
14A17Y	MLSALARPAGAAL<u>A</u>RS-<u>Y</u>STSAQNN-mature	*Pvu*I
14A17L	MLSALARPAGAAL<u>A</u>RS-<u>L</u>STSAQNN-mature	*Pvu*I
14A17S	MLSALARPAGAAL<u>A</u>RS-<u>S</u>STSAQNN-mature	*Pvu*I
14A18A	MLSALARPAGAAL<u>A</u>RS-F<u>A</u>TSAQNN-mature	*Hin*dIII, *Spe*I
14A19A	MLSALARPAGAAL<u>A</u>RS-FS<u>A</u>SAQNN-mature	*Hin*dIII, *Nhe*I

the importance of the basic amino acids in the extension peptide of pre-MDH for the processing, we constructed mutants with alanine substituted for arginine residues. First we mutated Arg^{14}, Arg^{15}, or both of them. The wild type and mutated precursors were *in vitro* transcribed and translated in the presence of [^{35}S]methionine. Radiolabeled precursors were incubated with bovine liver MPP at 30°C for 10 min, then the products were analyzed by SDS-PAGE and fluorography (Fig. 1). Processing efficiencies were calculated from the ratio of conversion of precursors to intermediate-MDH, after correction for the loss of the N-terminal methionine. Although we did not determine the processing site for the mutant precursors, we assume that the processing reactions occurred at a correct site because processed mutant MDHs migrated to the same extent as the wild type on the SDS-PAGE gel, and amino acid analysis confirmed that all peptide substrates were processed at a correct processing site. Nearly a half of wild type MDH was processed to the intermediate form in 10 min (Fig. 1A, lane 2). The 14A mutant was processed at a similar rate to the wild type (Fig. 1A, lane 3) and the 15A mutant was processed more slowly than the wild type (47% *versus* 27%, Fig. 1A, lane 4). Arg^{15} seems to be the more effective of the two consecutive proximal arginine residues. When both arginines were replaced by alanine, practically no processing of the mutant occurred (Fig. 1A, lane 5). These results indicate that MPP requires an arginine residue at one of the two position, proximal to the cleavage site, in agreement with the peptide substrate (*22*). Thus, the same mechanisms for recognition of the proximal arginine function for the peptide and the precursor. To confirm the role of Arg^{15}, we analyzed the mutant with lysine substituted at this position. We used the 14A mutant as a reference, since the processing of the 14A mutant was almost same with that of wild type as shown above. Introduction of lysine at position 15 caused a significant loss in processing (Fig. 1A, lane 6). This result indicates that Arg^{15} is not replaceable with lysine and that MPP requires both basicity and precise side-chain structure of the amino acid residue at position 15 to fit the binding site of MPP. These results are consistent with the recognition mechanism for the proximal arginine obtained by the peptide studies (*22*).

Replacement of Arg^{7} with alanine did not cause an apparent reduction of processing efficiency (Fig. 1B, lane 2). This result indicates that Arg^{7} is replaceable with alanine if the precursor protein contains two consecutive arginines at positions 14 and 15, which is different from the result obtained with the peptide substrate (*22*). When both Arg^{7} and Arg^{14} were replaced with alanine, little processing occurred in the resultant 7A14A mutant (Fig. 1B, lane 3). Since the 14A mutant was processed at a similar rate to the wild type, the substitution of Arg^{7} should have reduced the processing efficiency toward the 7A14A mutant. This result shows evidence that Arg^{7} functions as the distal arginine. In addition, comparison of the processing efficiencies of the 7A mutant and the 7A14A mutant, suggests that even Arg^{14} could serve partially as a distal arginine, since introduction of alanine at position 14 of the 7A mutant resulted in loss of processing. From these results, we suppose that the distal arginine residue is redundant and its position is not required to be fixed and at least one of these arginine residues is sufficient for the processing.

We also substituted lysine for Arg^{7} to examine the role of arginine. Replacement of Arg^{7} of the 14A mutant with lysine had no apparent effect on the processing efficiency compared with that of the 14A mutant (Fig. 1A, lane 3; Fig. 1B, lane 4). This result indicates that Arg^{7} is replaceable with lysine, and that MPP apparently recognizes them as a basic amino acid residue at the distal portion of the extension peptide. When we replaced both Arg^{7} and Arg^{15} with lysine, the 7K14A15K mutant was not processed as well as the 14A15K mutant (Fig. 1B, lane 5).

Together, these results showed evidence that MPP requires a basic amino acid residue in the distal portion of the extension peptide of the precursor protein. This requirement is also demonstrated in the case of peptide substrates (*22*). However, the contribution of Arg^{7} as the basic amino acid to the recognition signal is somewhat different between the precursors and the peptides. It is possible that the mature portion might exert structural effects on the extension peptide and cause different results between the precursor and the peptide, but this remains speculation. Nevertheless, we suppose that the processing of the precursor represents the processing in the mitochon-

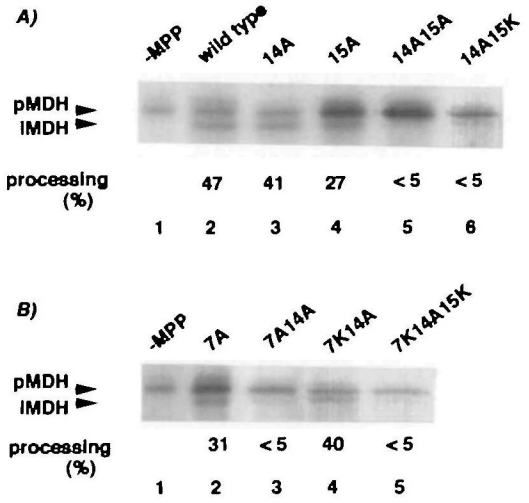

Fig. 1. **Processing of wild type and arginine-replaced mutants.** Radio-labeled precursors were incubated with bovine liver MPP for 10 min at 30°C. Processed products were separated by SDS-PAGE, visualized by fluorography, and quantified using NIH image. See "EXPERIMENTAL PROCEDURES" for details. Mutations are in the proximal region (A), and distal region (B).

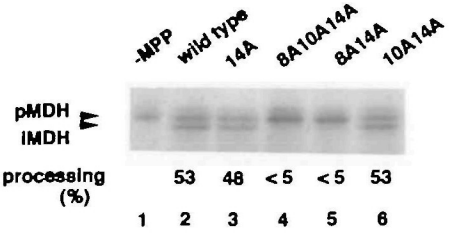

Fig. 2. **Processing of wild type and proline- and glycine-replaced mutants.** Radio-labeled precursors were incubated with bovine liver MPP for 10 min at 30°C. Processed products were separated by SDS-PAGE, visualized by fluorography, and quantified using NIH image. See "EXPERIMENTAL PROCEDURES" for details.

dria better than does the processing of peptide, since the precursor protein should contain all the information to be processed by MPP.

Contribution of Proline and Glycine between Proximal and Distal Arginine Residues—Although some mitochondrial proteins are not processed after translocation into mitochondria, their N-terminal sequences show some resemblance to extension peptides of mitochondrial precursor proteins; several arginine residues are present among their neutral amino acids. The difference in primary structure between the cleavable and the non-cleavable precursors is the poverty of proline and glycine residues in the N-terminal portion of the latter. An NMR study revealed that the non-cleavable mitochondrial proteins had a continuous α-helix at the N-terminal portion (*27, 28*). Another NMR study showed that deletion of a RPG sequence between the 10th and 14th arginine residues of aldehyde dehydrogenase precursor led to formation of a single continuous α-helix over the cleavage point and resulted in the loss of processing (*29, 30*). These results suggest that formation of the continuous α-helix through the extension peptide interferes with the processing reaction of MPP. Previously, using the peptide substrates, we obtained evidence that α-helix-breaking amino acid residues in the extension peptide of MDH affect the processing by MPP (*24*). Replacement of both Pro^8 and Gly^{10} resulted in reduction of the processing, suggesting that at least one of proline and glycine is required for the efficient processing. To analyze the roles of these proline and glycine residues in processing of the precursor, we replaced Pro^8 and/or Gly^{10} with alanine and examined the processing efficiency toward these mutants. As demonstrated above, Arg^{15} is critical as the proximal arginine since the 14A mutant was effectively processed at a comparable rate to the wild type. To examine the essential recognition element in the extension peptide of pre-MDH, we used the 14A mutant as a reference and constructed the following mutants carrying an alanine residue at position 14. Replacement of both proline and glycine with alanine (8A10A14A mutant) resulted in a significant reduction in the processing efficiency (Fig. 2, lane 4). To elucidate which amino acid residue is the more critical for the processing, we constructed mutants carrying a single substitution of proline (8A14A mutant) or glycine (10A14A mutant). Little processing was observed for the 8A14A mutant, and essentially no change occurred in the 10A14A mutant as compared with the 14A mutant (Fig. 2, lanes 5 and 6). These data indicate that proline is a critical amino acid for efficient processing. However, it is unclear whether an α-helix region exists in the extension peptide of MDH and, if so, whether proline residue really breaks the α-helix region.

Preference for the Amino Acids at P_1' Site—Sequence analysis of the two-step processed precursors revealed that phenylalanine, leucine, valine, and isoleucine are usually situated at the P_1' site of MPP cleavage (*15*). Isaya *et al.* demonstrated that such amino acids residues at the P_1' site would serve as an important recognition signal for MIP (*13*). In the study using the model peptides, we found that MPP had a preference for the aromatic and hydrophobic amino acid residues at the P_1' site (*24*). We examined whether these amino acids at the P_1' site were also effective for the processing of the pre-MDH by MPP. When phenylalanine at the P_1' site of pre-MDH was substituted for tyrosine, the resultant 14A17Y mutant gave a processing efficiency comparable with the Phe-type 14A mutant (Fig. 3, lane 4). The processing efficiencies of alanine and leucine mutants were lower than that of the Phe-type 14A mutant (lanes 5 and 6), and that of the serine mutant was very poor (lane 7). For the peptide substrates, the relative activities of tyrosine, leucine, alanine, and serine mutants were about 50, 20, 15, 5% of the wild type, respectively. The preference for amino acid at the P_1' site obtained by use of peptide substrates corresponds well with that of precursors. Thus, we confirm that the amino acid at the P_1' site is also an important determinant for processing of substrates by MPP. The results also indicate that the binding pocket of MPP for the amino acid of the P_1' site is large and its surface is hydrophobic, although this remains unclear until information on the tertiary structure of MPP is available.

MPP Also Recognizes P_2' and P_3' Sites—We also analyzed the effects of amino acid residues downstream from the cleavage site on the processing. We replaced amino acids at the P_2' and P_3' sites with alanine. The replacements had significant effects on the processing. Little processing product was detected for the P_2' mutant (14A18A), and the processing efficiency of the P_3' mutant (14A19A) was reduced to one half of that of the wild type (Fig. 4, lanes 4 and 5). Therefore, amino acid residues at both P_2' and P_3' sites are also important for the processing, indicating that MPP recognizes amino acid residues both at the cleavage site and downstream from cleavage site. This is the first demonstration of the significance of these amino acid residues for the processing. Since the N-terminal portions of the octapeptide are rich in hydrophilic, especially hydroxylated amino acids, a hydrophilic environment at the C-terminal region from the cleavage site should be present in the substrates to achieve efficient processing.

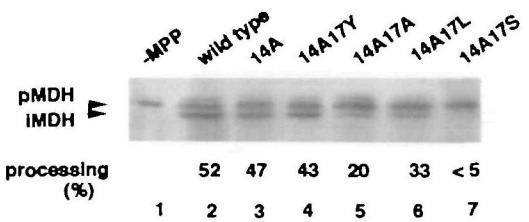

Fig. 3. **Processing of wild type and P_1' mutants.** Radio-labeled precursors were incubated with bovine liver MPP for 10 min at 30°C. Processed products were separated by SDS-PAGE, visualized by fluorography, and quantified using NIH image. See "EXPERIMENTAL PROCEDURES" for details.

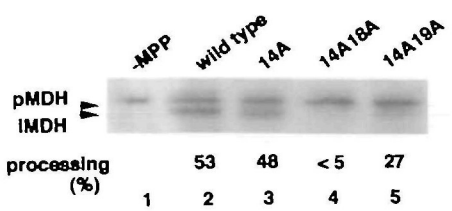

Fig. 4. **Processing of wild type and P_2' and P_3' mutants.** Radio-labeled precursors were incubated with bovine liver MPP for 10 min at 30°C. Processed products were separated by SDS-PAGE, visualized by fluorography, and quantified using NIH image. See "EXPERIMENTAL PROCEDURES" for details.

Further analysis is, however, required to elucidate the common nature of amino acids at these sites for the processing.

In conclusion, essentially the same recognition mechanisms function for the model peptides and precursor proteins. Therefore, it is confirmed that the processing assay employing synthetic peptides is valid for analyzing the processing reaction. However, there was some discrepancy between the results from these substrates, which might be due to structural effects of the mature portion. Thus, effective combinations of model peptides and precursor proteins are needed for the study of substrate-recognition mechanisms of MPP: model peptides are suitable for kinetic and systematic analysis of the processing, while precursor proteins are practical for qualitative analysis and for confirmation of the processing mechanism obtained by use of the peptide substrates.

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