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Importance of Residues Carboxyl Terminal Relative to the Cleavage Site in Substrates of Mitochondrial Processing Peptidase for Their Specific Recognition and Cleavage

Myeong-Cheol Song,[1] Tadashi Ogishima,[2] and Akio Ito

Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka 812-8581

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We previously identified distal and proximal arginine residues in the N-terminal portion and an aromatic amino acid at position 1 (P_1' site[3]) relative to the cleavage site as important recognition signals in substrates of mitochondrial processing peptidase [Niidome, T., Kitada, S., Shimokata, K., Ogishima, T., and Ito, A. (1994) *J. Biol. Chem.* 269, 24714-24722; Ogishima, T., Niidome, T., Shimokata, K., Kitada, S., and Ito, A. (1995) *ibid.* 270, 30322-30326]. To further elucidate the elements required for the specific recognition and cleavage by the enzyme, we synthesized synthetic peptides that possessed only the distal and proximal arginine residues and phenylalanine at the P_1' site in a poly alanine sequence, and analyzed the processing reaction toward them. They were not cleaved by the peptidase although they inhibited the peptidase activity. However, when serine was introduced into the C-terminal portions of the sequence, processing was observed. The efficiency of the resultant peptides improved as the number of serine residues was increased. A peptide with serine or histidine at P_2' and threonine at P_3' was processed most efficiently. These results indicate that the processing reaction catalyzed by the peptidase depends not only on the N-terminal portion but also on the C-terminal portion from the cleavage site in the substrates.

Key words: mitochondrial-import, mitochondrial-protein-degradation, peptidase, processing-protease, substrate-recognition.

Most mitochondrial proteins are encoded by nuclear genes and synthesized as larger precursors bearing an N-terminal extension peptide that is responsible for targeting to the mitochondrial matrix. In the matrix, the extension peptides are cleaved by mitochondrial processing peptidase (MPP) (*1-3*). For precursors that are cleaved in two steps, the initial step is catalyzed by MPP, and mitochondrial intermediate peptidase (MIP) subsequently cleaves the resultant octapeptide from the intermediate (*4*). MPP is a metallopeptidase and forms a heterodimer consisting of two structurally related α- and β-subunits, both of which are indispensable for processing (*5*). MPP has been purified from *Saccharomyces cerevisiae, Neurospora crassa*, rat liver, potato tubers, and spinach leaves (*6-10*).

The activity of MPP is strictly specific to mitochondrial precursor proteins; other proteins including mitochondrial mature proteins are not recognized by MPP. Analysis of a large number of amino acid sequences of the extension peptides has revealed no consensus sequence for the processing, except that basic amino acid residues, usually arginine, are often found at position -2 or -3 relative to the cleavage site (*11-13*). Using synthetic peptides modeled on the extension peptides of rat malate dehydrogenase (MDH) and ornithine aminotransferase (OAT), we have recently demonstrated that a set of two basic amino acids in the peptide, a proximal arginine at position -2 relative to the cleavage site, and a distal basic amino acid at about -10 are necessary for effective processing (*14, 15*). We have also shown that MPP exhibits considerable preference for aromatic and, to a lesser extent, hydrophobic amino acids at position 1 (*16*). In addition to this preference of MPP for amino acids at definite positions, some higher-order structure in the extension peptides has been reported to be needed for specific recognition by MPP. Other structural features of the extension peptides are a potential ability to form an amphiphatic α-helix and the presence of helix-breaking amino acids, like glycine and proline, between the tentative distal and proximal arginine residues. From an NMR study on synthetic peptides corresponding to the extension peptides of mitochondrial protein precursors, Hammen *et al.* (*17*) showed that peptides to be cleaved by MPP formed a helix-linker-helix structure, in which glycine and/or proline residues served as an α-helix-breaking linker.

[1] Present address: Baker Laboratory of Chemistry, Cornell University, Ithaca, NY 14853-1302, USA.

[2] To whom correspondence should be addressed. Tel/Fax: +81-92-642-2530, E-mail: taogiscc@mbox.nc.kyushu-u.ac.jp

[3] The nomenclature of Schechter and Berger [Schecher, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27, 157-162] is used to describe the interaction between substrate and enzyme. The amino acids in the substrate are designated as P_1', P_2', P_3',..., P_i', in the C-terminal direction and P_1, P_2, P_3,..., P_i in the N-terminal direction from the bond undergoing cleavage.

Abbreviations: MPP, mitochondrial processing peptidase; MIP, mitochondrial intermediate peptidase; Fmoc, *N*-α-9-fluorenylmethoxycarbonyl; MDH, malate dehydrogenase; OAT, ornithine aminotransferase.

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Although most studies, including ours (*14-16*), on recognition elements responsible for the processing reaction by MPP have focused on the N-terminal portion relative to the cleavage site, since MPP is absolutely specific to the precursor proteins, some reports have also suggested involvement of the carboxy-terminal portion in the recognition and cleavage by MPP (*18-20*). In a study of the processing signals for MIP, Isaya *et al.* (*21*) found that mutations in the octapeptide of pre-MDH prevented cleavage of the precursor by MPP.

In the present study, in order to determine whether the carboxy-terminal portion relative to the cleavage site of the precursor proteins is really important in their recognition and cleavage by MPP, we synthesized model peptides having various amino acids at positions 2, 3, and 4 in addition to the so-far-elucidated recognition signals at fixed positions, *i.e.*, arginine at positions −2, −5, and −8 and phenylalanine at position 1. Kinetic studies using the synthetic peptides as the substrates of MPP revealed that amino acids at positions 2 and 3 were also important for the processing reaction.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—All the synthetic peptides were synthesized by an EPS 221 peptide synthesizer (ABIMED, Langenfeld, Germany) using the *N*-α-9-fluorenylmethoxycarbonyl (Fmoc) strategy (*22*). Peptides were purified by reversed-phase HPLC (Cosmosil AR-5C18, 10×250 mm; Nacalai Tesque, Kyoto), and elution was established with an acetonitrile gradient. Eluted peptides were monitored by the absorbance at 215 nm and collected manually. Purity of the peptides was verified by mass spectrometry (SX102A JEOL; Tokyo).

Assay of Processing Activity—The peptides were tested for cleavage by MPP, which had been purified from bovine liver as described by Ou *et al.* (*7*). Reactions were performed and stopped as described previously (*15*). Briefly, the peptide substrates and 5 or 10 μl of purified MPP (0.8 μM) were incubated in a final volume of 100 μl of 20 mM Hepes-KOH buffer (pH 7.4) containing 0.1% of Tween 20 at 30°C for 5-10 min. After the incubation, the reaction was stopped by addition of 1 mM EDTA. Reaction products were analyzed by the HPLC system equipped with a data processor (Chromatopack C-R7A; Shimadzu, Kyoto). The reaction mixture was injected onto an ODS column (Cosmosil AR-5C18, 4.6×150 mm; Nacalai Tesque). Elution was carried out with a linear gradient established between 0 and 50% acetonitrile in 0.05% trifluoroacetic acid for 40 min. The flow rate was 0.7 ml/min, and the products were detected by monitoring the absorbance at 215 nm. The fragments generated by MPP were identified by reference to standard synthetic peptides. Each assay was run at least in triplicate, and mean value was determined. The data from these experiments were plotted using the Lineweaver-Burk procedure and the corresponding plots proved to be linear.

TABLE I. **Inhibitory effects of peptides with minimum recognition elements.** The reaction mixture contained varying concentrations of synthetic peptides and a fixed concentration (5 μM) of a substrate, MDH [1-21]. The inhibitory activity was determined and is given as the residual activity in the presence of added peptide (20 μM). The cleavage site is indicated by an arrowhead, and tentative recognition elements are shown in bold. Experiments were performed at least three times as described in "EXPERIMENTAL PROCEDURES."

Peptide	Sequence	Residual activity (%)
	▼	
1	ARAAARA FAAAA	95
2	ARAAAAARA FAAAA	92
3	ARAARAARA FAAAA	80
4	ARAAARAAARA FAAAA	45

RESULTS

Processing Activity toward Peptides with Minimum Recognition Elements—Our first question was whether the recognition elements that we previously elucidated (*14-16*), *i.e.*, an arginine residue at −2 (proximal position), a positively-charged residue at position about −10 (distal position), and an aromatic or hydrophobic amino acid residue at position 1 (P_1'), are sufficient for processing by MPP. To resolve this, we synthesized peptides containing arginine residues at the proximal and distal positions and phenylalanine at position 1 in a polyalanine sequence (peptides 1 and 2 in Table I). None of these peptides was cleaved at a measurable rate (data not shown), indicating that the above signals alone were not sufficient for processing by MPP. We also synthesized two other model peptides that contained one more arginine residue in another distal portion (peptides 3 and 4 in Table I) since we previously found that an extension peptide of OAT needed two distal basic amino acids for efficient processing by MPP (*15*). They were also not cleaved by MPP either. They did, however, show inhibitory effects toward the processing of the extension peptide of MDH (Table I). Peptide 4 at 20 μM significantly inhibited processing of MDH [1-21] (extension peptide of MDH from 1 to 21) by 55% while peptides 1, 2, and 3 exerted little effect on the processing. Peptide 4 could thus at least be recognized, though not cleaved, by MPP. These results indicated that peptides with only the distal and proximal arginine residues and phenylalanine at P_1' do not have sufficient recognition signals for their processing by MPP, although these elements are necessary for the specific binding of extension peptides to the enzyme.

A comparison of amino acid sequences of the C-terminal portion from the cleavage site among extension peptides of mitochondrial precursor proteins reveals an abundance of hydroxyl amino acids, especially serine residues. We

TABLE II. **Kinetic constants for synthetic peptides with Ser or Thr in the C-terminal portion.** The cleavage site is indicated by an arrowhead, and substituted residues are shown in bold. The peptide with the sequence ARAARAARAFAAAA is the reference peptide. Experiments were performed as described in "EXPERIMENTAL PROCEDURES."

Sequence	K_m (μM)	V_{max} (pmol·min^{-1})	K_m/V_{max}
▼			
ARAARAARA FAAAA	−[a]	<0.3	−[a]
ARAARAARA FSAAA	29.6±12.1	1.1±0.3	0.04
ARAARAARA FASAA	21.5±4.5	1.4±0.2	0.06
ARAARAARA FAASA	42.5±9.1	1.0±0.3	0.02
ARAARAARA FSSAA	9.5±1.7	3.0±0.4	0.3
ARAARAARA FSSSA	8.6±1.5	4.8±1.3	0.6
ARAARAARA FSTSA	3.1±0.3	20.4±0.7	6.6

[a]Not determinable due to the low activity.

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therefore introduced one residue of serine instead of alanine in the C-terminal portion of the model peptide (peptide 4 in Table I). Substitution of serine for alanine at position 2, 3, or 4 rendered the peptides cleavage-competent by MPP though the catalytic activity was very low and the K_m value was high (Table II). The processing efficiency (V_{max}/K_m) improved when the number of the serine residues was increased. Nevertheless, the processing efficiency toward the peptide with three serine residues at positions 2-4 was still only about one-tenth of that toward the peptide that had the C-terminal portion of the extension peptide of pre-MDH (FSTSA). The only difference between these two peptides was that the latter possessed threonine instead of serine residue at the third position from the cleavage site.

Amino Acids at Positions 2 and 3 Affect Processing Efficiency—As shown above, the model peptide having FSTSA as the C-terminal portion from the cleavage site was most efficiently cleaved by MPP. To identify which amino acids in this region are required for efficient processing, the amino acids at positions 2, 3, and 4 (S, T, and S, respectively) were individually substituted by alanine (Table III). Replacement of the serine at position 2 with alanine resulted in about a two-fold increase in the K_m value and in a marked reduction of V_{max} to about 1/20 that of the control. Replacement of threonine with alanine at position 3 caused about a 10-fold increase in K_m and a more than 10-fold decrease in V_{max}, with the result that the processing efficiency was dramatically reduced to less than 1/100 that of the control peptide. In contrast, the kinetic parameters were little affected by substituting alanine for serine at position 4. These results indicated that the amino acids at positions 2 and 3 are more important for the efficient cleavage of the peptide.

To further elucidate the roles of the residues at positions 2 and 3, we synthesized a series of peptides that had various amino acids at these positions (Tables IV and V). Serine and threonine are most abundant as the second residue relative to the scissile bond in mitochondrial precursor proteins, and sometimes histidine is found at this position. We thus substituted threonine and histidine for serine (Table IV). The enzymatic activity toward the threonine-substituted peptide was reduced in terms of V_{max} to about 1/20 that of the reference peptide, while the histidine-substituted peptide was cleaved more rapidly (about 2-fold) than the reference one. The enhanced cleavage of the histidine-substituted peptide seemed not to be due to requirement by MPP for a basic amino acid at this position because the reaction toward the arginine-substituted peptide had a low V_{max} value.

We next examined the effects of amino acid replacements at position 3 (Table V). Substitution of serine, cysteine, glutamine, and valine for threonine resulted in slightly increased K_m values and in dramatic decreases in V_{max} values. Substitution of serine for threonine caused about a 10-fold reduction of the processing efficiency, while the other substitutions brought about much greater reductions (about 70-fold). These results indicated that the amino acid residue at position 3 also participates in the efficient cleavage of the substrates. Since the peptide with threonine-serine at positions 2 and 3 was not cleaved as efficiently as that with serine-threonine at these positions, the sequence of the residues appears to be important.

To determine the amino acid preference of MPP with respect to positions 2 and 3 for efficient processing, we analyzed a model peptide that had five amino acids derived from the corresponding region of cytochrome b_2 precursor at the carboxy-terminus (Table VI). MPP exhibited a higher K_m and lower V_{max} toward the peptide, so the

TABLE III. **Effects of substitutions in the C-terminal portion relative to the cleavage site on processing by MPP.** The cleavage site is indicated by an arrowhead, and substituted residues are shown in bold. The peptide with the sequence ARAARAARAFSTSA is the reference peptide. Experiments were performed as described in "EXPERIMENTAL PROCEDURES."

Sequence	K_m (μM)	V_{max} (pmol·min^{-1})	K_m/V_{max}
ARAARAARA ▼ FSTSA	3.1±0.3	20.4±0.7	6.6
ARAARAARA FATSA	7.4±0.3	1.2±0.1	0.16
ARAARAARA FSASA	29.4±5.5	1.6±0.1	0.05
ARAARAARA FSTAA	3.8±12.1	16.6±3.4	4.4

TABLE IV. **Effects of Ser at position 2 relative to the cleavage site on processing by MPP.** The cleavage site is indicated by an arrowhead and substituted residues are shown in bold. The peptide with the sequence ARAARAARAFSTSA is the reference peptide. Experiments were performed as described in "EXPERIMENTAL PROCEDURES."

Sequence	K_m (μM)	V_{max} (pmol·min^{-1})	K_m/V_{max}
ARAARAARA ▼ FSTSA	3.1±0.3	20.4±0.7	6.6
ARAARAARA FTTSA	8.7±2.5	1.0±0.1	0.1
ARAARAARA FRTSA	5.0±0.5	0.8±0.3	0.2
ARAARAARA FHTSA	4.5±1.0	40.0±2.7	8.9

TABLE V. **Effects of substitutions for Thr at position 3 relative to the cleavage site on processing by MPP.** The cleavage site is indicated by an arrowhead, and substituted residues are shown in bold. The peptide with the sequence ARAARAARAFSTSA is the reference peptide. Experiments were performed as described in "EXPERIMENTAL PROCEDURES."

Sequence	K_m (μM)	V_{max} (pmol·min^{-1})	K_m/V_{max}
ARAARAARA ▼ FSTSA	3.1±0.3	20.4±0.7	6.6
ARAARAARA FSSSA	8.6±1.5	4.8±1.3	0.6
ARAARAARA FSCSA	17.3±5.4	0.8±0.1	0.05
ARAARAARA FSNSA	15.1±3.0	0.9±0.1	0.06
ARAARAARA FSVSA	16.5±4.1	1.9±0.4	0.1
ARAARAARA FTSSA	10.3±0.3	1.0±0.1	0.1

TABLE VI. **Effects of substitutions in the C-terminal portion from the cleavage site by MPP.** The cleavage site is indicated by an arrowhead, and substituted residues are shown in bold. The peptide with the sequence ARAARAARAFSTSA is the reference peptide. Experiments were performed as described in "EXPERIMENTAL PROCEDURES."

Sequence	K_m (μM)	V_{max} (pmol·min^{-1})	K_m/V_{max}
ARAARAARA ▼ FSTSA	3.1±0.3	20.4±0.7	6.6
ARAARAARA YGSTA	10.1±2.3	1.5±0.1	0.1
ARAARAARA YSSTA	10.8±2.1	5.2±0.4	0.5
ARAARAARA YGTTA	9.8±4.5	2.2±0.6	0.2
ARAARAARA YSTTA	7.9±2.7	24.6±2.4	3.1

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cleavage efficiency was only about 1/50 of that with the FSTSA sequence. When glycine and serine at positions 2 and 3 were replaced with serine and threonine, respectively, MPP cleaved the resultant peptide with an efficiency about half that of the peptide with the FSTSA sequence.

Cytochrome b_2 precursor is also a substrate of MPP. We did not, however, determine if pre-cytochrome b_2 was cleaved by MPP much less efficiently than pre-MDH or whether they were cleaved at similar rates. If the latter is the case, the C-terminal portion with the native N-terminal portion would be a better combination for cytochrome b_2 precursor for efficient processing by MPP.

DISCUSSION

Peptides having a polyalanine sequence with only the recognition elements that we have previously elucidated were not cleaved by MPP, although some inhibitory effects toward the MPP reaction were observed. Such peptides did, however, become cleavable by MPP if they had hydroxyl amino acids at position 2, 3, or to a lesser extent at position 4. Alternation of the amino acid at position 2 had a great effect on the V_{max} value rather than on the K_m, value indicating that the residue at this position is involved in a process in the cleavage reaction. On the other hand, in the case of position 3 the catalytic activity was influenced greatly, as well as the K_m value to a lesser extent.

MPP preferred histidine or serine at position 2 and threonine at position 3 in its substrates when they had the ARAARAARAF immediately before the positions. We have thus presented evidence that in addition to these previously elucidated recognition elements, the amino acids C-terminal from the MPP-cleavage site, especially positions 2 and 3, are also critical for the processing of precursor peptides by MPP. Analysis of the frequency of amino acids at positions 2 and 3 in a sample of 52 mitochondrial extension peptides revealed a high frequency of serine (46%) and, to a lesser extent, of histidine (17%) at position 2, while threonine (30%) and serine (21%) occurred most frequently at position 3 (*11-13*). Thus, the statistical data support our experimental results.

With respect to the pairing of amino acids at positions 2 and 3, the order of processing efficiency was HT>ST>SS>RT, TT, TS, SV>SC>SN>AT>SA>AA. Preference for a specific pairing could indicate that MPP recognizes amino acids at positions 2 and 3 as a single subsite. Alternatively, the enzyme may interact with each of the amino acids at these positions independently, since interaction between the side chains of the two amino acids leading to such a pairing preference cannot be ruled out.

The common property of the three amino acids (His, Ser, and Thr) preferred by MPP at positions 2 and 3 is their ability to form hydrogen bondings. In some way, they are able to adopt the conformation needed for efficient processing, and the substrate and enzyme can interact with each other through hydrogen bonding. A small hydrophobic interaction may be involved, since threonine was more effective than serine at position 3. A suitable fitting of positions 2 and 3 in addition to the proximal arginine and phenylalanine or tyrosine at position 1 of the substrates would facilitate the correct and rapid cleavage of the scissile bond.

The proximal arginine and an aromatic amino acid residue (generally phenylalanine) at position 1 are known to be essential for the cleavage of precursors by MPP. We have established in this study that the amino acids at positions 2 and 3 are also important for the MPP reaction. Thus, the sequence RXFH(S)T should be regarded a newly determined strong cleavage motif for MPP.

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