J. Biochem. 126, 874-878 (1999)

Analysis of Recognition Elements for Mitochondrial Processing Peptidase Using Artificial Amino Acids: Roles of the Intervening Portion and Proximal Arginine[1]

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Received July 27, 1999; accepted August 23, 1999

We recently demonstrated, using synthetic peptides modeled on the extension peptide of malate dehydrogenase, that amino acid residues present at the proximal and distal positions relative to the cleavage site are critical determinants for the recognition of substrates by mitochondrial processing peptidase [Niidome *et al.* (1994) *J. Biol. Chem.* 269, 24719-24722). While the proximal arginine is unexceptionally located at the −2 position, the position of the distal residue varies among mitochondrial precursor proteins. Between the proximal and distal residues, proline and/or glycine are present in most mitochondrial precursor proteins, and they are considered to play a role in the specific recognition of a substrate by the peptidase. To elucidate the role of the intervening portion, we introduced a non-natural amino acid [2-(2-aminoethoxy)acetic acid] between the distal and proximal residues. We also analyzed the functional elements in the proximal arginine by replacing the residue with various arginine or lysine analogs. The results of kinetic studies indicated that the intervening portion should be flexible for efficient processing, and that the guanidino group of the proximal arginine is recognized by the peptidase through hydrogen and ionic bonds.

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

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Most mitochondrial proteins are synthesized as precursor proteins on cytosolic polysomes and are subsequently imported into mitochondria. Many precursors carry an amino-terminal extension peptide, which contains information for their targeting to mitochondria. The extension peptides of the imported proteins are proteolytically removed after translocation of the precursors across the mitochondrial membranes (*1-3*). The extension peptides are usually removed in one step by mitochondrial processing peptidase (MPP) (*4, 5*); some of them are removed in sequential steps by two independent peptidases. Precursors with such extension peptides are initially processed by MPP to intermediate forms, and then the intermediates are sequentially cleaved by mitochondrial intermediate peptidase (MIP) (*6*).

MPP has been purified from *Neurospora crassa* (*7, 8*), *Saccharomyces cerevisiae* (*9*), potato tubers (*10*), and spinach leaves (*11*), and rat liver mitochondria (*4, 5, 12*). The purified enzymes are inhibited by divalent cation-chelators such as EDTA and *ortho*-phenanthroline. MPP is highly specific for mitochondrial precursor proteins; other proteins including mitochondrial mature and secretory proteins are not recognized by MPP. This indicates that the extension peptides of mitochondrial precursor proteins should contain information for recognition by MPP. They however, do not have consensus amino acid sequences for processing, except that basic amino acid residues, usually arginine, are often observed at the −2 or −3 position relative to the cleavage site (*3, 12-14*). Using synthetic peptides modeled on the extension peptide of rat malate dehydrogenase (MDH), we have recently shown the importance of two arginine residues in peptide substrates; one is present at −2 and the other at −10 from the cleavage site (*15*). We designated the former as the proximal and the latter as the distal residue. We also assigned an aromatic or hydrophobic amino acid at the P_1' position[3] as another recognition element (*16*). The presence of arginine as the proximal residue at −2 was almost a prerequisite for a MPP substrate, whereas that at the distal position, which should only be basic, varied. Essentially the same results were obtained for peptides modeled on ornithine amino-

[1] This work was supported in part by a Grant-in-Aid for Scientific Research, No. 08458200 (A.I.), and a Grant-in-Aid for Scientific Research, No. 07680692 (T.O.), from the Ministry of Education, Science, Sports and Culture of Japan.

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[3] In accordance with Schecheter and Beger (1967), the enzyme binding sites are denoted as S_1, $S_2\cdots$, S_i and S_1', $S_2'\cdots$, S_i' away from the scissile bond toward the N- and C-terminis, respectively. Amino acid residues in the substrates are referred to as P_1, P_2,$\cdots$, P_i and P_1', $P_2'\cdots$, P_i in accordance with the binding site.

Abbreviations: MPP, mitochondrial processing peptidase; MIP, mitochondrial intermediate peptidase; MDH, malate dehydrogenase; ALDH, aldehyde dehydrogenase; Fmoc, *N*-α-9-fluorenylmethoxy-carbonyl; HPLC, high performance liquid chromatography; AEA, 2-(2-aminoethoxy)acetic acid.

transferase (17), and also for precursor proteins of MDH (18).

Proline and/or glycine are present between the tentative distal and proximal residues in most mitochondrial precursor proteins. Substitution of alanine for these proline and glycine residues reduced the cleavage efficiency for the model peptide of MDH whereas the cleavage efficiency for the peptides with the distal arginine at various positions did not significantly change as long as the peptides had the proline and glycine residues (16). Using model peptides of rat aldehyde dehydrogenase precursor with the RGP sequence between the tentative distal and proximal residues, Thornton *et al.* (19) demonstrated that a peptide without the RPG formed a continuous single α-helix, while the native peptide showed two discontinuous helices separated by the sequence. From these results, they concluded that the proline and/or glycine residues acted as helix-breaking residues in the extension peptide, making the peptide cleavage-competent. They also showed that model peptides for matrix proteins that are not proteolytically processed upon import formed a continuous α-helical structure (20). Their data together with ours strongly indicate that the intervening portion between the distal and proximal residues plays some important role in the processing of the extension peptides.

To further analyze the structural requirement for the recognition of substrates by MPP, we investigated the processing reaction using the synthetic peptides, especially from the following viewpoints. (i) What is the role of the intervening portion? (ii) Why does the proximal residue have to be arginine? To answer these questions, we synthesized substrate peptides with non-natural or artificial amino acids at the positions of interest, and analyzed the kinetic reactions. The results indicated that peptides whose proximal and distal residues were connected not through normal peptide-bonds but through flexible and modestly hydrophilic ether bonds were efficiently processed. We also demonstrated that the guanidino group of the proximal arginine was recognized by MPP through hydrogen and ionic bonds.

EXPERIMENTAL PROCEDURES

Preparation of Synthetic Peptides—Peptides were synthesized manually or with an Economy Peptide Synthesizer (ABiMED Analysen-Technik GmbH, Langenfield, Germany) employing the N-α-9-fluorenylmethoxycarbonyl (Fmoc) solid-phase method (21). After synthesis, the peptides were deprotected and cleaved from the resin with trifluoroacetic acid/trimethylsilyl bromide as described (22). The peptides were purified by reverse-phase high performance liquid chromatography (HPLC) on a Cosmosil AR-5C18 column (10×250 mm; Nacalai Tesque, Kyoto). The purity of the peptides was confirmed by PICO-TAG™ or FAB-Mass. Peptide concentrations were determined by the ninhydrin method (23). Fmoc-amino acids were purchased from Watanabe Chemical Industries (Hiroshima), NOVABIOCHEM® (Läufelfingen, Switzerland), and BACHEM einchemikalien AG (Bubendorf, Switzerland).

Assaying of Processing Peptidase Activity—The enzyme reaction was performed with the synthetic peptides and purified MPP in 0.4 ml of 20 mM Hepes-KOH buffer (pH 7.3) containing 0.1% Tween 20 at 30°C. MPP was isolated from bovine liver mitochondria according to the established method (4). The reaction was terminated by the addition of 0.1 ml of 0.25% trifluoroacetic acid after an appropriate time. The mixture was centrifuged at 10,000×g for 5 min, and then the supernatant was analyzed by HPLC on a Cosmosil 5C18-AR column (4.7×150 mm; Nacalai Tesque) equilibrated with 1% acetonitrile containing 0.05% trifluoroacetic acid. Elution was carried out with a linear gradient established between 1 and 36% acetonitrile in 0.05% trifluoroacetic acid for 30 min. The substrate and product peptides were detected by monitoring at 215 nm. Michaelis constants (K_m) and maximum velocities (V_{max}) were determined from a Lineweaver-Burk plot of the initial velocities against the substrate concentrations. The kinetic values shown in the tables are the averages of three to five determinations.

Synthesis of Fmoc-2-(2-aminoethoxy)acetic Acid—A solution containing 10 mmol (1.05 g) of 2-(2-aminoethoxy)-ethanol (Wako Pure Chemical Industries, Osaka) dissolved in 10 ml of dimethoxyethane was dropped slowly into 15 mmol (5.05 g) of Fmoc-N-hydroxysuccinimide ester dissolved in 50 ml dimethoxyethane. The reaction was allowed to continue with stirring overnight, and then the product was analyzed by thin-layer chromatography. The plate was developed with chloroform:methanol:acetic acid (95:10:3). After completion of the reaction, the solution was evaporated to dryness under reduced pressure. The dried residues, mostly Fmoc-aminoethoxyethanol, were then oxidized to the corresponding carboxylic acid essentially by an established method involving pyridinium dichromate (24). The oxidant (35 mmol) dissolved in 25 ml N,N-dimethylformamide was added to the dried residue. The mixture was stirred for 7-9 h at 25°C and then diluted 7-fold with water (300 ml). Ethyl acetate (200 ml) was added to the mixture, and the aqueous phase was separated and extracted with ethyl acetate (3×200 ml). The organic phases were combined and dried with magnesium sulfate. The solvent was evaporated off. The dried residue was dissolved in a small amount of ethyl acetate, and then the resulting solution was loaded onto a silica gel 60G column (4.5×4 cm), which had been equilibrated with ethyl acetate. After washing extensively with ethyl acetate, the column was eluted with chloroform:methanol:acetic acid (50:50:1). The eluate was dehydrated with magnesium sulfate and then evaporated to dryness under reduced pressure. The production of Fmoc-2-(2-aminoethoxy)acetic acid was confirmed by FAB-Mass.

RESULTS AND DISCUSSION

Insertion of ω-Amino-Fatty Acids between the Proximal and Distal Residues—We used MDH1-21, which corresponds to the first to 21st amino acids of the extension peptide of MDH, as the control peptide. In this peptide, the peptide bond between Ser^{16} and Phe^{17} is cleaved as well as that in the native precursor protein. All other synthetic peptides used in this study were cleaved at this point. Although the K_m value for MDH1-21 (see Table I) was slightly lower than that we reported previously (15), the value was constant throughout the present study. The ionic concentration, probably potassium ions, which was present in the final preparation of MPP, dramatically affected the K_m value as well as the V_{max} value. Even a low concentra-

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TABLE I. **Effects of insertion of AEA molecules between the distal and proximal arginine residues in peptides.** The distal and proximal ariginine residues of MDH1-21 are connected with one to five repeats of the AEA molecule. The data are means±SE obtained for three to five repeats. See "EXPERIMENTAL PROCEDURES" for details.

	Substrate	K_m (μM)	V_{max} (pmol·min^{-1})	V_{max}/K_m
MDH1-21	MLSALARPVGAALRRS-FSTSA	0.13±0.09	27.6±5.2	212
MDH8A10A	MLSALARAVAAALRRS-FSTSA	0.23±0.06	13.5±2.3	58.7
MDH(AEA)$_1$	MLSALAR(AEA)$_1$ RRS-FSTSA	0.07±0.04	37.8±8.9	540
MDH(AEA)$_3$	MLSALAR(AEA)$_3$ RRS-FSTSA	0.09±0.07	42.0±9.5	467
MDH(AEA)$_5$	MLSALAR(AEA)$_5$ RRS-FSTSA	0.28±0.15	42.3±11.3	151

tion of potassium (*e.g.*, a final concentration of 10 mM) significantly increased the K_m value. The removal of potassium ions was unsuccessful due to the tendency of MPP to aggregate during dialysis. Therefore, we tried to use an enzyme preparation with a high concentration to minimize the effect of the ions brought into the reaction medium.

We supposed that the intervening portion did not necessarily have to consist of normal peptide-bonds if its role is only to make the backbone flexible or to break a continuous helix in the extension peptides. Then, we attempted to determine the roles of the intervening sequence by introducing ω-amino-fatty acids with various carbon chain lengths [NH_2-$(CH_2)_n$-COOH] between the proximal and distal arginines. Although the reaction with the peptide connected with β-alanine ($n=2$) occurred slowly in a concentration-dependent manner ($K_m=0.2$ μM and $V_{max}=$ 8.6 pmol/min), the reactions with other peptides ($n=3$-10) did not obey Michaelis-Menten's equation; significant inhibition was observed at high concentrations (data not shown). Possibly, they failed to form a correct structure for the substrate or formed aggregates due to the hydrophobicity of the middle portion.

Insertion of 2-(2-Aminoethoxy)acetic Acid between the Proximal and Distal Arginine Residues—In order to avoid the undesirable hydrophobicity, we introduced oligomers of 2-(2-aminoethoxy)acetic acid (AEA) to determine whether or not the intervening region can function when it is very flexible. An AEA molecule has an ether linker and does not have a side chain. These properties are suitable for the analysis of flexibility since we can minimize the number of peptide bonds and avoid the undesirable hydrophobicity. The Fmoc-amino acid derivative was synthesized as described under "EXPERIMENTAL PROCEDURES." The peptide without the insertion ($n=0$) was not cleaved by MPP. The insertion of one repeat of AEA [MDH-(AEA)$_1$] made the peptide cleavage-competent. The K_m value was lower than and the V_{max} value higher than those of the control peptide, MDH1-21, therefore the processing efficiency was more than twice that in the case of the control peptide (Table I). Three repeats of AEA [MDH-(AEA)$_3$)] gave essentially the same processing efficiency as that with MDH-(AEA)$_1$. MPP showed the highest processing efficiency as to these peptides among the synthetic peptides we have synthesized to date.

The lengths of one, three and five repeats of AEA would correspond to those of two, six, and ten natural amino acid residues, respectively. Compared with peptides of the same length, MDH1-21, MDH8A10A, and MDH-(AEA)$_3$, the peptide with AEA was cleaved two times more efficiently than MDH1-21 and about eight times more than MDH8A10A. Since an AEA molecule lacks both a side chain and a peptide bond, it exhibits greater flexibility than a normal dipeptide. Thus, the peptides with AEA molecules are possibly more flexible than the control peptide between the distal and proximal arginine residues.

We suppose that a kind of substrate-enzyme induced-fit should occur when MPP recognizes and cleaves the extension peptides, since they have a random structure in an aqueous environment (*19*). The critical determinants for the substrates are at least a distal basic amino acid, a proximal arginine, whose position is indispensably at −2, and an aromatic or hydrophobic amino acid at the P_1' position. The peptide having the proximal and distal arginines connected only with PVG was cleaved at almost the same rate by MPP (*16*), which indicated that the distance between the proximal and distal residues was changeable. This was also proved by the present study since the peptides with (AEA)$_1$ and (AEA)$_3$ were almost equally and efficiently cleaved, and even that with (AEA)$_5$ was cleaved with a high V_{max} value. These data suggest that although the distance between the proximal and distal residues varies in the peptide chain, these amino acids are spatially close to each other in the enzyme-substrate complex. The intervening portion with flexibility could facilitate the approach of the distal basic amino acid to the acceptor site in MPP, which may exist close to the binding sites of the proximal arginine and P_1' amino acid. As a result, an induced-fit between the critical elements and the substrate-binding points in MPP occurs. Then, the carbonyl oxygen on the scissile bond would come to the fourth (or fifth when the activated hydroxide is considered) coordinate of the zinc ion, and finally the carbon would be attacked by the activated hydroxide ion.

The insertion of one or three repeats of the AEA molecule increased the k_{cat} and decreased the K_m values compared with those for the reference peptide. The latter parameter, however, seemed to increase as the number of inserted amino acids increased. The peptide that had five repeats of AEA [MDH-(AEA)$_5$] showed a 2-fold increase in the K_m value, whereas it still exhibited a V_{max} value similar to that of MDH-(AEA)$_3$. Thus, the cleavage efficiency tended to decrease owing to the increase in the K_m value as the distance between the distal and proximal residues increased. If the simple assumption that K_m correlates with K_s could be made, a higher K_m value for MDH-(AEA)$_5$ indicates that the connection with a long chain hampers the binding or fitting of the peptide to MPP. Otherwise, substrates merely become larger for the binding-pocket. Anyway, a connecting region of appropriate length and probably with flexibility is necessary between the proximal and distal residues for efficient cleavage of extension peptides by MPP.

Proline and glycine residues are present at high fre-

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quencies in mitochondrial extension peptides, especially between the tentative proximal and distal residues. Our results together with those of NMR studies support the concept that the function of glycine and proline residues between the distal and proximal residues is to give a portion of extension peptides flexibility, which makes the precursors processing-competent or at least more apt to be cleaved.

Substitution of the Proximal Arginine by Arginine or Lysine Analogs—We then asked what are the essential elements in the proximal arginine for the proximal recognition signal. To answer this, we synthesized a series of peptides that had analogous amino acids at position −2 instead of arginine, and then analyzed their cleavage efficiencies. In this experiment, the arginine residue at position −3 was substituted with alanine to exclude its influence. The peptide, which has a single arginine residue at position −2, was used as a reference peptide. It was cleaved with almost the same kinetic parameters as MDH1-21. Table II shows that all the substitutions reduced the processing efficiency to varying degrees. Substitution of lysine for arginine gave a 3-fold increase in K_m and a 3-fold reduction in V_{max}, so that the V_{max}/K_m value became one-tenth of that of the reference peptide. The value was, however, the best for all peptides tested that had other amino acids than arginine at the proximal position. This indicates that a positive charge is an important requisite for the proximal amino acid. The substitution of *N*-nitroarginine, where the nitro group bound to the ω-nitrogen in the guanidino side chain group, and that of citrulline, where C=NH was replaced by C=O, gave 20- and 70-fold reductions in the V_{max}/K_m value, respectively. Although both analogues do not have a positive charge, the ω- and δ-amino, and imino groups of *N*-nitroarginine, and the ω- and δ-amino groups of citrulline can be hydrogen-donors in hydrogen bonds. The processing efficiency as to the peptide with the former analogue was 3-fold higher than that as to the peptide with the latter one. As discussed below, the δ-nitrogen of the guanidino group seems not to be involved in the specific recognition of the proximal residue. Thus, hydrogen bonds through the terminal nitrogen of the guanidino group should also be important factors for the specific recognition of the proximal residue. The processing efficiency as to the peptide with nitroarginine was next to that for the peptide with lysine. This suggests that the side chain of the analogue could partially compensate for the lack of a positive charge through hydrogen bonds. *N*-ε-Acetyllysine is also an analogue of arginine, whose NH_2 is substituted by CH_3 and C=NH by C=O. The peptide with this amino acid showed a 300-fold reduction in the cleavage efficiency. This indicates that the nitrogen of the side chain of *N*-ε-acetyllysine, which could correspond to the δ-nitrogen of the guanidino group of arginine, cannot be a hydrogen-donor for the specific recognition of the proximal residue. Although *N*-ε-acetyllysine has an additional carbon atom relative to arginine, this seemed to have less effect on the processing since almost the same processing efficiency was observed for the peptide with *N*-ε-acetylornithine (data not shown). MPP showed almost no cleavage activity toward the peptide with *N*-ε-trimethyllysine, although this amino acid possesses a positive charge. Due to the bulkiness of the trimethyl group, the amino acid at the −2 position could not enter the acceptor site for the proximal residue (S_2 site) in the substrate-binding pocket of MPP. Otherwise, *N*-ε-trimethyllysine was accommodated in the S_2 site but in such a way that other parts failed to establish productive (to be cleaved at the scissile bond) binding with MPP. In conclusion, we suppose that MPP would recognize the proximal arginine at least through the ionic and hydrogen bonds.

The peptide with alanine was cleaved with a high K_m value but a relatively high V_{max} value, which was next to that of the reference peptide. Since this amino acid can form neither an ionic nor a hydrogen bond, the interaction of the side chain with the S_2 site should be very small if it occurs. In other words, due to the small size of the side chain, the alanine can be accommodated in the S_2 pocket with the aid of other recognition signals but is not fixed at the site. A relatively high V_{max} value of the peptide with alanine suggests that once it enters the substrate-binding pocket its fixing at the P_2 site is not mandatory for the following correct and efficient processing. We suppose that the proximal amino acid residue would be primarily necessary for the efficient binding of extension peptides and that its correct binding determines the position of the scissile bond with the aid of binding between the P_1' and S_2' sites. We recently obtained the data showing that the amino acids at the +2 and +3 positions (P_2' and P_3') are also

TABLE II. **Effect of replacement of the proximal arginine with an analogous amino acid.** The proximal (−2) arginine of a derivative of MDH1-21, whose −3 arginine was replaced with alanine, was substituted for by an analogous amino acid. The data are means±SE obtained for three to five repeats. See "EXPERIMENTAL PROCEDURES" for details.

Amino acid at −2 position	K_m (μM)	V_{max} (pmol·min⁻¹)	V_{max}/K_m
L-Arginine	0.15±0.03	25.3±6.3	169
L-Lysine	0.52±0.05	7.5±0.17	14.4
N-ω-Nitro-L-arginine	0.89±0.17	6.6±0.33	7.4
L-Citrulline	1.8±0.2	4.4±0.06	2.4
L-Alanine	7.8±1.4	10.4±2.0	1.3
N-ε-Acetyl-L-lysine	7.4±3.2	3.6±1.4	0.5
N-ε-Trimethyl-L-lysine	3.9±2.0	1.6±0.4	0.4

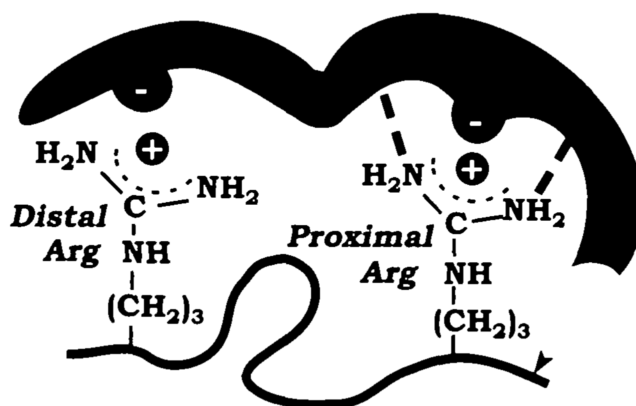

Fig. 1. **Schematic mode of recognition of the distal and proximal arginine residues by MPP, and the possible role of a "flexible-linker."** These residues are recognized through an ionic bond, and a combination of hydrogen and ionic bonds, respectively. They could be accommodated in the binding pocket of MPP regardless of their distance owing to the flexible-linker. The arrowhead indicates the cleavage site.

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important signals for the processing by MPP (*25*). Thus, other amino acids than the proximal arginine can also contribute to the binding of peptides to the active site. In the case of alanine, the peptide could form a "correct" structure without the binding of a P_2 amino acid. In contrast, all the amino acids with bulky side chains (N-ω-nitroarginine, citroline, N-ε-actetyllysine, and N-ε-trimethyllysine) except arginine reduced the catalytic rate. Possibly, these side chains could play a role in the binding of substrates and MPP at the P_2-S_2 sites, but should shift the scissile bond away from the active center of MPP.

In this study, we obtained data indicating that a flexible region between the distal and proximal arginine residues is necessary for the efficient processing of precursor proteins, and also that the guanidino group of the proximal arginine is recognized by MPP through hydrogen and ionic bonds (Fig. 1). Such a study could only be performed by using artificial amino acids in combination with synthetic peptides, *i.e.* not by using *in vitro*-translated precursor proteins.

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