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Role of Basic Amino Acids in the Cleavage of Synthetic Peptide Substrates by Mitochondrial Processing Peptidase

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Our recent experiments using model peptides of rat malate dehydrogenase (MDH) indicated that a proximal arginine and a distal basic amino acid are important for processing by mitochondrial processing peptidase (MPP). [Niidome, T., Kitada, S., Shimokata, K., Ogishima, T., and Ito, A. (1994) *J. Biol. Chem.* 269, 24719-24722]. To elucidate if the recognition elements apply to other precursor proteins, we analyzed cleavage of model peptides of human ornithine aminotransferase (OAT). Purified peptidase cleaved peptides that corresponded to N-terminal 1-25 and 3-25 at the correct site (Gly^{17}-Val^{18}) at nearly equal rates. Replacement of Arg^{16} (−2 position) with lysine or alanine reduced the processing efficiency by 95- and 380-fold, respectively. Either deletion from Met^{1} to Arg^{10} or replacement of the basic amino acids between them decreased the processing efficiency considerably. A peptide containing Arg^{7} in addition to Lys^{4} and Arg^{10} was more effective than the control peptide. However, a peptide with one and two consecutive basic amino acids in the distal region had a processing efficiency close to the control peptide. These results indicated that processing of OAT was enhanced by an increase in the number of basic amino acids with a suitable distance between them. In other respects, the processing signal of OAT was essentially the same as that of MDH.

Key words: mitochondrial processing peptidase, substrate specificity.

Most mitochondrial proteins are nuclearly encoded and synthesized with N-terminal extension peptides that contain sufficient information for their targeting and localization to mitochondria. They are selectively imported into mitochondria, where the extension peptides are proteolytically removed by a matrix-localized mitochondrial processing peptidase (MPP) (*1-3*). For precursors that are cleaved in two steps, the N-terminal main part of the extension peptide is first removed by MPP, then the remaining octapeptide is cleaved by mitochondrial intermediate peptidase (*4*). MPP has been purified from *Saccharomyces cerevisiae* (*5*), *Neurospora crass* (*6*), rat liver (*7*), potato tuber (*8*), and spinach mitochondria (*9*). The enzyme consists of two structurally related proteins, α-MPP and β-MPP, and is highly specific for mitochondrial precursor proteins. Other proteins, including mitochondrial mature proteins and secretory protein precursors, are not recognized by MPP. This indicates that the extension peptides of mitochondrial precursor proteins should have information for recognition by MPP.

Although MPP does not recognize a specific amino acid sequence, the extension peptides of mitochondrial precursor proteins have certain characteristic features. Most notably, an arginine residue is often observed at −2 or −3 position from the cleavage site. This arginine residue is conserved in many mitochondrial precursors from various species. Site-specific mutagenesis of the human ornithine transcarbamylase extension peptide has shown that the arginine residue important for transport of the peptide into mitochondria and for its proteolytic processing to the mature form (*10*). Experiments with synthetic peptides modeled on the extension peptide of rat malate dehydrogenase (MDH) have also shown the importance of the arginine residues together with a basic amino acid residue that was distant from the cleavage site (*11, 12*).

In this study, to elucidate whether the recognition elements found in the extension peptide of MDH apply to other precursor proteins, we synthesized various oligopeptides corresponding to the amino acid sequence of the extension peptide of human ornithine aminotransferase (OAT) and analyzed the cleavage reaction by MPP. The results obtained were the same as those with MDH, except that OAT needed the presence of more than two distal basic amino acids with a suitable distance between them.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Synthetic peptides were manually synthesized by the *N*-α-9-fluorenylmethoxycarbonyl solid-phase method (*14*). The completed peptides were deprotected and cleaved from the resin with trifluoroacetic acid/trimethylsilyl bromide (*15*). The peptides were purified by reversed-phase high performance liquid chromatography (HPLC) on a Cosmosil AR-5C18 column (10×250 mm; Nacalai Tesque, Kyoto), and their purity was confirmed by amino acid analysis.

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Abbreviations: MPP, mitochondrial processing peptidase; OAT, ornithine aminotransferase; MDH, malate dehydrogenase; HPLC, high performance liquid chromatography.

Assay of Processing Activity—Peptides were tested with a purified preparation of MPP, which had been isolated from bovine liver mitochondria according to Ou *et al.* (*7*). The standard assay was essentially the same as the method reported by Niidome *et al.* (*11*). 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, and the mixture was subjected to HPLC analysis. The column (Cosmosil 5C18-AR, 4.6×150 mm, Nacalai Tesque) was developed at a flow rate of 0.7 ml/min with a linear gradient of acetonitrile (0-50%) in 0.05% trifluoroacetic acid for 30 min. The substrate and product peptides were detected by monitoring absorbance at 215 nm. The identities and amounts of the hydrolytic products were determined by comparison with the retention times and peak areas of standard peptides. Fragments generated by MPP were also identified by amino acid analysis. Values of K_m and V_{max} were determined from Lineweaver-Burk plots of initial velocity against substrate concentration. The kinetic parameters shown in the tables were averages of three to five determinations.

RESULTS

Cleavage of a Synthetic Peptide—Figure 1 shows the sequences of the extension peptides of human OAT and rat MDH. The peptide corresponding to human OAT was synthesized. The synthetic peptide was incubated with the purified MPP, and the reaction products were separated by HPLC. The chromatogram gave only three fragments when monitored at 215 nm. These fractions were collected and analyzed for amino acid composition. The first and second peaks were the reaction products, Met^1-Gly^{17} and Val^{18}-Ala^{25}, respectively (data not shown). The third peak corresponded to the substrate. These results indicated that the cleavage occurred only at the correct site between Gly^{17} and Val^{18}.

Influence of Basic Residues at the N-Terminal Portion of Pre-OAT for Efficient Processing—To investigate which portions of the extension peptide of OAT are important for efficient processing by MPP, we synthesized a series of peptides with successive deletions of N-terminal amino acids. We measured initial velocities of the cleavage and evaluated K_m and V_{max} from Lineweaver-Burk plots (Fig. 2). OAT[3-25], which had the first two amino acids deleted from OAT[1-25], gave a K_m of 8.4±2.6 μM, which was close to that for OAT[1-25], and a V_{max} that was a little higher than that for OAT[1-25] (Table I). When six residues were deleted from the N-terminus, processing efficiency, V_{max}/K_m, was reduced about 10-fold. A more pronounced effect on these kinetic parameters was observed when ten residues were deleted. For OAT[11-25], K_m value increased about 8-fold and V_{max} value decreased about 8-fold, and therefore, the V_{max}/K_m value dramatically decreased about 63-fold. The change in the kinetic parameters of the synthetic peptides seemed to be correlated with the loss of three basic residues, Lys^4, His^7, and Arg^{10}. Thus, a decrease in the processing efficiency appeared to be the result of elimination of a positive charge rather than simple reduction of the peptide length, as was expected from our previous results (*11, 12*).

To further analyze the roles of positive charges in the N-terminal portion, we examined derivatives of a control peptide, OAT[3-25], in which the basic residues on the N-terminal portion of OAT were replaced by alanine residues. Table II shows that OAT[K4A], in which lysine at position 4 was replaced by alanine, showed essentially the same K_m value as the control peptide, whereas its V_{max} value was reduced 4-fold. The substitution in OAT[R10A] also produced little effect on the K_m value. The double substitution in OAT[K4A/R10A] gave a K_m value higher than that of the control peptide, while the V_{max} value was reduced about 4-fold. These results indicated that the

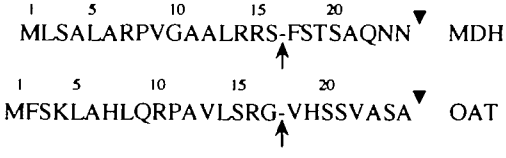

Fig. 1. **N-terminal amino acid sequences of the pre-MDH and pre-OAT.** MPP cleavage sites are indicated by arrows. Sites of cleavage by mitochondrial intermediate peptidase are indicated by arrowheads.

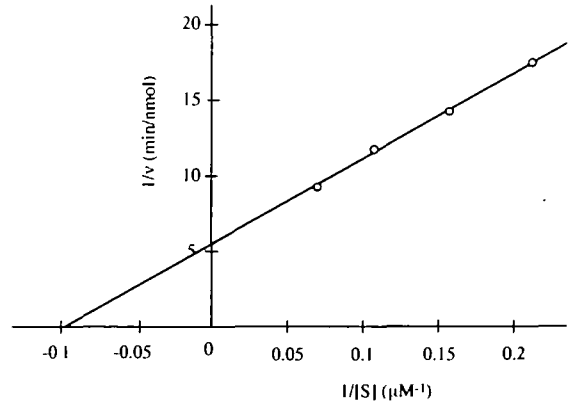

Fig. 2. **Lineweaver-Burk plot of reaction rate *versus* concentration of substrate.** The substrate was OAT[1-25]. Kinetic constants were estimated by fitting the data to the Michaelis-Menten equation by the method of least squares.

TABLE I. **Processing of synthetic peptides of various lengths by MPP.** Kinetic parameters of the processing were calculated from Lineweaver-Burk plots. Positively charged amino acids are underlined, and the cleavage site is indicated by "-."

Peptides	Sequence	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m
OAT[1-25]	MFSKLAHLQRPAVLSRG-VHSSVASA	10.2±1.1	149±21.3	15
OAT[3-25]	SKLAHLQRPAVLSRG-VHSSVASA	8.4±2.6	157±25.5	19
OAT[7-25]	HLQRPAVLSRG-VHSSVASA	10.6±1.2	60± 7.9	6.0
OAT[9-25]	QRPAVLSRG-VHSSVASA	17.8±5.9	34±14.8	2.0
OAT[11-25]	PAVLSRG-VHSSVASA	64.4±4.5	20± 7.0	0.3

TABLE II. **Effect of substitution of the distal basic amino acid residues on processing by MPP.** Substituted residues are shown in bold, and the cleavage site is indicated by "-."

Peptides	Sequence	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m
OAT[3-25]	SKLAHLQRPAVLSRG-VHSSVASA	8.4±2.6	157±25.5	19
OAT[K4A]	SALAHLQRPAVLSRG-VHSSVASA	8.8±1.2	41± 1.9	5.0
OAT[R10A]	SKLAHLQAPAVLSRG-VHSSVASA	10.6±2.9	60± 6.3	7.0
OAT[K4A/R10A]	SALAHLQAPAVLSRG-VHSSVASA	19.0±2.4	39±13.9	2.0
OAT[H7A]	SKLAALQRPAVLSRG-VHSSVASA	11.2±2.1	151±17.4	14
OAT[H7R]	SKLARLQRPAVLSRG-VHSSVASA	10.4±1.5	297±35.3	29
OAT[Q9R]	SKLAHLRRPAVLSRG-VHSSVASA	8.2±0.9	148±33.1	18

TABLE III. **Effect of substitution of the arginine residue at position −2 from the cleavage site on processing by MPP.** Substituted residues are shown in bold, and the cleavage site is indicated by "-."

Peptides	Sequence	K_m (μM)	V_{max}/K_m (pmol/min)	V_{max}
OAT[3-25]	SKLAHLQRPAVLSRG-VHSSVASA	8.4±2.6	157±25.5	19
OAT[R][R16K]	SKLAHLQRPAVLSKG-VHSSVASA	19.4±1.9	4± 0.3	0.2
OAT[R16A]	SKLAHLQRPAVLSAG-VHSSVASA	20.8±2.0	1± 1.6	0.05

presence of at least one of the two basic amino acid residues, was sufficient for a low K_m value, and that the presence of both was necessary for a high V_{max} value.

The processing efficiency of OAT[H7A] was close to that of OAT[3-25], which indicates that the histidine at position 7 makes little or no contribution to substrate recognition as a distal basic amino acid. This, however, seems to contradict the data in Table I that suggest the role of His^7 as a distal basic amino acid. Although we cannot fully explain this discrepancy, it is possible that His^7 can substitute for Lys^4 in the absence of this residue. The lack of function of His^7 as a distal basic amino acid is evident from the kinetic parameters of OAT[H7R], in which the residue was replaced by arginine. The V_{max} value of this peptide increased twofold over the control peptide. In contrast, OAT[Q9R], which had one lysine residue at position 4 and two arginine residues at positions 9 and 10, had a processing efficiency at the control level. There was no abnormal cleavage of either OAT[H7R] or OAT[Q9R], *i.e.*, processing was always between Gly^{17} and Val^{18}. The difference in the kinetic data between OAT[H7R] and OAT[Q9R] suggests that the catalytic rate of MPP increases as the number of the distal basic amino acid residues that are located with a suitable distance each other increases.

Requirement of an Arginine Residue at Position −2 from the Cleavage Site—An earlier study indicated that the arginine residue at position −2 rather than position −3 was necessary for the recognition and cleavage of the extension peptide of MDH (*11*). To examine the role of the arginine residue in the case of OAT, the arginine at position −2 was replaced by alanine or lysine. Both the substitutions reduced processing efficiency (Table III). Substitution of the arginine with lysine increased K_m value about 2-fold and markedly decreased V_{max} value approximately 40-fold, so that V_{max}/K_m value became 1/80 of that of the control peptide. Moreover, substitution of alanine caused a 2-fold increase in the K_m and a 160-fold reduction in the V_{max} value. These results indicated that the arginine residue at −2 position was indispensable not only for processing of pre-MDH but also for that of pre-OAT. Thus, the arginine residue seems to be a primary determinant in the recognition and processing by MPP.

DISCUSSION

MPP is responsible for proteolytic cleavage of the extension peptides of mitochondrial precursor proteins. The extension peptides have signals not only for targeting of precursor proteins to the mitochondria but also for their specific cleavage in the matrix, although there is no sequence homology among the extension peptides of different protein species. They are abundant in arginine, serine, leucine, and alanine residues, but poor in acidic residues. Our previous studies (*11, 12*) on processing by MPP using model peptides of the extension peptide of MDH have indicated that there are two sets of critical basic residues: one arginine at a position −2 from the cleavage site and one basic amino acid (lysine or arginine) distant from (about ten residues) the cleavage site. The proximal arginine residue could be a primary determinant for substrate-recognition and position of cleavage. The distal basic amino acid seems to promote the processing. The importance of basic amino acids in extension peptides has also been shown using recombinant precursor proteins. For example, either deletion of various parts of the extension peptide of pre-adrenodoxin or replacement of arginine residues in the middle portion with neutral amino acids resulted in a drastic decrease of the processing efficiency (*13*).

In this study, we have focused on the role of the basic residues in the extension peptide of OAT. Processing of the synthetic peptides with N-terminal deletions revealed that an N-terminal portion of the extension peptide that contained two basic amino acid residues was required for the efficient reaction. A large change in K_m value occurred when ten residues were deleted. The change in kinetics of processing correlated with the loss of two basic residues, Lys^4 and Arg^{10}. Support for the contribution of the basic residues was apparent from the data for OAT[K4A/R10A]. In the case of MDH, one distal arginine residue was necessary for processing, whereas two or more basic amino acid residues were required for efficient processing in the case of OAT. Since OAT[H7R], which had Lys^4, Arg^7, and Arg^{10}, showed the highest V_{max} value of the peptides examined, the number of distal basic amino acids clearly contributed to the processing efficiency. However, an increase in total net charge alone did not improve the

processing efficiency since OAT[Q9R], which contained two consecutive basic amino acid residues, had essentially the same efficiency as the control peptide. The presence of basic amino acids with a suitable distance between them was important.

Replacement of the arginine at −2 position of the control peptide by lysine gave a remarkable increase in K_m and a decrease in V_{max}. Replacement of the arginine by alanine made the peptide virtually non-cleavable. These observations indicated that, in addition to basicity of the residue, the side-chain structure of arginine was critical for processing of the extension peptides. In both cases, processing occurred only at the correct site. No processing between Pro^{11} and Ala^{12} was observed, indicating that Arg^{10} could not substitute for the proximal arginine. Portions located C-terminally from the cleavage site are also probably important for the cleavage. We have previously showed that the amino acid at P_1' was very important for efficient processing of the peptide substrates modeled for MDH (*12*).

Elements required for OAT processing were the same as those required for MDH, except that the processing efficiency of OAT improved as the number of distal basic amino acids with a suitable distance between them increased. Why the numbers of distal amino acids required for efficient processing differ is not presently clear. One explanation could be that the modeled MDH peptides do not show maximal processing efficiency. It is possible that MPP would cleave the MDH peptide more efficiently if the peptide had an additional basic amino acid in the distal portion. Thus, the combination of the proximal arginine and the distal basic amino acids would constitute a major part of the general processing signal, at least for precursors that undergo two-step cleavage.

We do not know the secondary structure, not to mention the tertiary one, of the model peptides since they do not adopt a secondary structure in an aqueous environment (*16, 17*). However, MPP seems to recognize an overall structure of the extension peptide rather than a short consensus sequence around the cleavage site. Thus, a common structure that is induced in substrate by MPP appears to be responsible for the specific binding and correct cleavage of extension peptides. Our experiments in combination with structural studies should contribute to clarification of the recognition mechanism of MPP.

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