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Recognition of Mitochondrial Protein Precursor Lacking Arginine at Position –2 by Mitochondrial Processing Peptidase: Processing of Bovine Cytochrome P450(SCC) Precursor[1]

Katsuhiko Kojima, Eiki Yamasaki, Sakae Kitada, Tadashi Ogishima, and Akio Ito[2]

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

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Mitochondrial processing peptidase (MPP) specifically cleaves off the N-terminal presequence of the mitochondrial protein precursor. Previous studies demonstrated that Arg at position –2 from the cleavage site, which is found among many precursors, plays a critical role in recognition by MPP. We analyzed the structural elements of bovine cytochrome P450 side-chain cleavage enzyme precursor [pre-P450(SCC)], which has Ala at position –2, for recognition by MPP. Replacement of Ala position –2 of pre-P450(SCC) with Arg resulted in an increase in the cleavage rate. Replacement with Gly caused a reduction in the cleavage rate and the appearance of an additional cleavage site downstream of the authentic site. A pre-P450(SCC) mutant with Met at position –2 retained cleavage efficiency equal to that of the wild type. These results indicate that –2 Ala of pre-P450(SCC) is recognized by MPP as a determinant for precise cleavage, and that the amino acid at –2 is required to have a straight methylene chain for interaction with the S_2 site. The preference for distal basic residues, a hydrophobic residue at +1, and hydroxyl residues at +2 and +3, was almost the same as those of the precursors with Arg at –2, indicating that the recognition mechanism of pre-P450(SCC) by MPP is essentially the same as that of the precursors with Arg at position –2.

Key words: mitochondrial processing peptidase, mitochondrial protein precursor, presequence, processing, substrate recognition.

Most nuclear-encoded mitochondrial proteins are translated on cytosolic ribosomes as precursor proteins carrying an N-terminal extension presequence. The presequences contain information for specific targeting of mitochondria. During or after import of the precursors into the matrix of mitochondria, the presequences are initially cleaved off by mitochondrial processing peptidase (MPP) (*1–4*). MPP is a metalloendopeptidase and forms a hetrodimer consisting of two structurally related subunits, α- and β-MPP. β-MPP is a catalytic subunit with the metal-binding motif HxxEH (*5, 6*), which is conserved in a pitrilysin superfamily (*7*). We recently demonstrated that some acidic residues of α-MPP participate in interaction with longer presequences (*8*), and that the conserved glycine-rich loop of α-MPP plays important roles in binding and cleavage of the precursor proteins (*9*). Thus, the enzyme activity requires functional cooperation between the two subunits.

There is no primary sequence homology around the cleavage sites among the many different precursors, though they are characterized by the presence of positively charged residues and the potential to form amphiphilic α-helices (*10*). The substrate recognition of MPP, however, is strictly specific. Gavel and von Heijne suggested four cleavage-site motifs of presequences of mitochondrial protein precursors by analyzing the N-terminals of mature proteins in databases (*11*). The precursors that are processed in one step by MPP share three motifs, XRX-XS (R-2), XRXY- (S/A) (R-3), and XXX-XS (R-none). Some precursors are initially processed by MPP, and residual octapeptides are subsequently removed by mitochondrial intermediate peptidase (*11*). These have the motif XRX- (F/I/L) SX (T/S/G) XXXX-X (R-10).

Earlier studies on substrate recognition of MPP have mostly been performed for R-2 and R-10 precursors. An arginine at position –2 from the cleavage site by MPP is considered as the most essential element for specific recognition (*12–15*). Basic amino acid residues distal to the cleavage site are required for effective cleavage (*12–16*). A flexible region containing proline and/or glycine often intervenes between the two basic residues (*15, 16*). Bulky hydrophobic residues, including aromatic ones, prefer the amino acid residue at position +1 (*14, 16*). Serine and threonine, and less often histidine and cystein, at positions +2 and/or +3 are critical for cleavage by MPP (*14, 17*).

On the other hand, the recognition mechanisms of MPP for R-3 and R-none are unknown. Most R-none precursors have yet to be confirmed as being cleaved by MPP. There-

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[2] To whom correspondence should be addressed. Tel: +81-92-642-2530, Fax: +81-92-642-2607, E-mail: a.itoscc@mbox.nc.kyushu-u.ac.jp

Abbreviations: MPP, mitochondrial processing peptidase; pre-COX IV, cytochrome *c* oxidase subunit IV precursor; pre-MDH, malate dehydrogenase precursor; pre-P450(SCC), cytochrome P450 side-chain cleavage enzyme precursor.

fore, there are possibilities of processing by an unknown protease, and trimming after cleavage at the authentic site by MPP.

In this paper, we analyzed the recognition elements of bovine cytochrome P450 side-chain cleavage enzyme precursor [pre-P450(SCC)], which is an R-none type that has an MPP cleavage site determined (*18*). Although pre-P450(SCC) lacks the most crucial structural element, arginine at position –2, the recognition mode by MPP is essentially the same as that of the R-2 precursors.

EXPERIMENTAL PROCEDURES

Construction of Mutant Proteins and In Vitro Synthesis—The cDNA of bovine cytochrome P450 side-chain cleavage enzyme precursor [pre-P450(SCC)] was isolated as reported previously (*19*). *Sal*I and *Xba*I restriction sites were then introduced at its 5′- and 3′-ends, respectively, by PCR, and the resultant DNA was inserted into a multiple cloning site of pSP64 vector. Mutagenesis was performed using QuickChange site-directed mutagenesis kit. Mutations were confirmed by DNA sequencing. Radiolabeled proteins were synthesized using the TNT SP6 coupled reticulocyte lysate system (Promega). According to the manufacturer's instructions, 1 μg of template plasmid was added to a reaction mixture containing 1.0 mCi/ml of [^{35}S]methionine (ICN Biomedicals) with rabbit reticulocyte lysate, and the mixture was incubated for 90 min at 30°C. Synthesized precursors were immediately used in MPP processing assay.

Purification of Recombinant Yeast MPP—Recombinant yeast MPP with a hexahistidine tag on the C terminus of α-MPP was expressed and purified as described previously (*8*). BL21(DE3) strain transformed with the expression vectors carrying cDNAs of α- and β-MPP was cultured for 24 h at 25°C, then harvested by centrifugation at 1,000 ×*g* for 10 min. After sonication of the harvested cells, the suspension was centrifuged at 10,000 ×*g* for 20 min. The resultant supernatant was loaded on a nickel-chelating Sepharose column (Amersham Pharmacia Biotech) equilibrated with buffer A (20 mM Hepes-KOH, pH 7.4, containing 500 mM NaCl, 30% glycerol, and 0.1% Tween 20). The column was washed with buffer A containing 50 mM imidazole. The hexahistidine-tagged proteins were eluted with buffer A containing 500 mM imidazole. The purity was confirmed by SDS-PAGE followed by Coomassie Blue staining.

Assay of Processing Peptidase Activity—The radiolabeled precursor proteins were incubated with purified MPP (25 ng) in 10 μl of 20 mM HEPES-KOH (pH 7.4) containing 1 mM $MnCl_2$ and 0.1% Tween 20 at 30°C. The reaction was terminated after an appropriate time by the addition of SDS to a concentration of 1%, and the processing products were separated by SDS-PAGE. The gels were then analyzed with a BAS 1500 bioimaging analyzer (Fuji). The cleavage efficiency was calculated as the percentage of the mature forms of P450(SCC) relative to the total amounts of protein.

RESULTS AND DISCUSSION

Alanine at Position –2 of Pre-P450(SCC) Is Recognized by MPP as a Determinant for Precise Cleavage—Bovine cytochrome P450 side-chain cleavage enzyme precursor [pre-P450(SCC)] lacks arginine at position –2, but other structural elements for recognition by MPP are present around the cleavage site, *i.e.*, basic amino acids in distal positions –9 to –11, a hydrophobic residue at position +1 and small hydrophilic residues in positions +2 and +3 (Fig. 1). Previous studies have shown that arginine at position –2 plays a crucial role in cleavage by MPP (*12–15*). Arginine at –2 is required not merely for its positive charge but for its guanidino group for interaction with the enzyme (*15*).

Recently, the crystal structure of yeast MPP complexed with pre-COX IV peptide was determined (*20*). The two subunits, α- and β-MPP, form a large cavity, the wall of which is lined with negatively charged and hydrophilic residues. The peptide is bound in an extended conformation at the active site in β-MPP, and forms main-chain hydrogen bonds with a β-strand neighboring the active site. The S_2 site containing βGlu160 and βAsp164 is located near the active site, and these acidic residues interact with the guanidino group of arginine at position –2 of the substrate.

It is clear that the structural elements around the cleavage site of pre-P450(SCC), a natural substrate of MPP, were not optimized for effective cleavage. Indeed, the substitution of arginine for alanine led to a more than 5-fold increase in the cleavage rate (Fig. 2B). This indicates that, with processing at the correct site, more effective cleavage would not be needed under physiological conditions.

We have demonstrated that multiple structural elements on the precursor proteins are involved in recognition by MPP (*21*). The substrate recognition mechanism, therefore, may permit a lack of arginine at position –2 in the presequence. We then investigated if alanine at position –2 of pre-P450(SCC) functions as a structural element for recognition by MPP. To verify the requirement of the methyl group of alanine, a G-2 mutant of pre-P450(SCC), which has glycine introduced into the –2 position, was constructed (Fig. 1). The cleavage rate of the G-2 mutant was reduced to about one-tenth of that of the wild type (Fig. 2C). In the cleavage of this mutant, an additional product with greater mobility than the wild type, though with low efficiency, was observed (Fig. 2C). This suggests that the introduction of glycine into this position weakens interaction with the S_2 site of MPP, and allows MPP access to another site in the substrate. For the additional cleavage site, we found the sequence PRPYS downstream of the authentic site, which matched the disposition of the recognition elements that we have investigated (*i.e.*, arginine at –2, a hydrophobic resi-

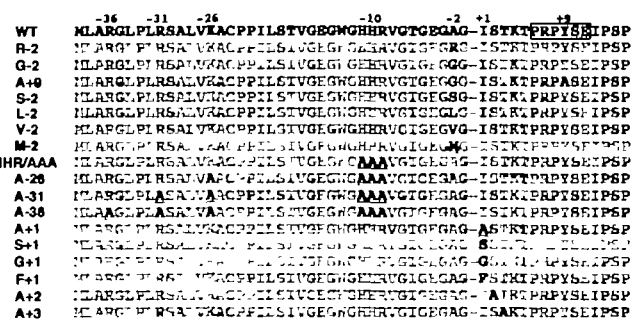

Fig. 1. **Amino acid sequences of the constructed mutants of bovine cytochrome P450 side-chain cleavage enzyme precursor.** Substituted amino acids are underlined. The hyphens indicate the authentic MPP cleavage sites. The boxed sequence indicates the R-3 motif one (see "RESULTS AND DISCUSSION").

due at +1, and serine at +2) (Fig. 1). The A+9 mutant, in which alanine was substituted for tyrosine at position +9 of the G-2 mutant (Fig. 1), was not cleaved at the downstream site but only at the authentic site (Fig. 2D), indicating that the additional cleavage of G-2 mutant occurs at this site. The PRPYS sequence also matches the R-3 cleavage motif, XRXY-S (*11*). It has been suggested that the R-3 motif would result from initial cleavage by MPP just before tyrosine, and the subsequent removal of the resulting N-terminal amino acid (*11, 22*). In the wild-type cleavage, the additional product was not observed (Fig. 2A). Proline residues around the downstream site may interfere with the formation of a β-strand of substrate bound to MPP (*20*). Therefore, the authentic site of the wild type would be preferentially recognized by MPP, even though it lacks arginine at position –2. Taken together, the results indicate that the alanine residue at position –2 of pre-P450(SCC) is recognized as a determinant for cleavage at the precise site, and that the methyl side-chain is involved in an interaction with the S_2 site of MPP.

The Straight Methylene Chain of Amino Acid Residue at Position -2 Contributes to Interaction with MPP—The requirement of the methyl group of alanine at position –2 led us to expect a hydrophobic interaction with the corresponding subsite in the enzyme. We further investigated the structural requirement of the amino acid at position –2. Serine, leucine, and valine residues were often found in mitochondrial presequences (*22*). The S-2 mutant showed an approximately 3-fold reduction of the cleavage rate, and additional cleavage was observed as in the G-2 mutant (Fig. 3A). This indicates that the hydroxyl side chain of serine is unfavorable for recognition through the hydrophobic interaction. L-2 and V-2 mutants were processed with the same efficiency as the S-2 mutant and also produced the additional product (Fig. 3, B and C). The branched side chains of leucine and valine appear to interfere with interaction with the S_2 site.

If such a steric restriction exists, then, methionine at position –2, which has a straight hydrophobic side chain, should be able to interact with the S_2 site. To confirm this possibility, we analyzed the M-2 mutant and found that it was cleaved at the single site at the same rate as the wild type (Fig. 3D). This result suggests the common interaction that occurs between amino acid at position –2 and the S_2 site. Because the side chain of arginine contains methylene groups, such an interaction, though weaker than an electrostatic one, would contribute to the recognition of R-2 type precursors. This hydrophobic interaction, in particular, is indispensable for recognition of pre-P450(SCC) by MPP.

Taylor *et al.* reported that in the crystal structure of the MPP-peptide complex, the S_2 site containing βGlu160 and βAsp164 is located near the main chain of the substrate, and therefore, the long side chain of arginine at position –2 bends from its β-carbon (*20*). They also reported that the guanidino group forms a salt bridge with βGlu160, while its methylene chain lies along the hydrophobic wall formed

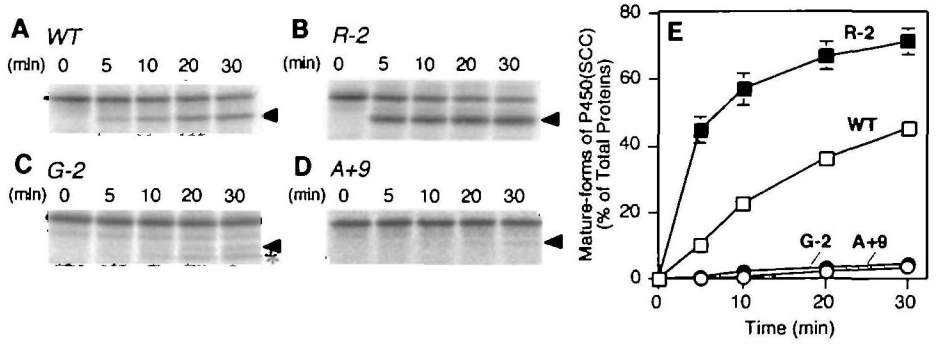

Fig. 2. **Recognition of alanine at position -2 as a determinant for precise cleavage.** A–D, time courses of cleavage of pre-P450(SCC) (WT) and the R-2, G-2, and A+9 mutants. Amino acid sequences of the precursors are represented in Fig. 1. *In vitro*–translated, [^{35}S]methionine-labeled pre-P450(SCC) and the mutants were incubated with purified yeast MPP (25 ng) in 10 μl of 20 mM HEPES-KOH (pH 7.4) containing 1 mM $MnCl_2$ and 0.1% Tween 20 at 30°C for the times indicated. The reactions were terminated by the addition of a final concentration of 1% SDS. All samples were subjected to SDS-PAGE, and the gels were analyzed using the BAS1500. The arrowheads indicate the mature forms of P450(SCC). The asterisk denotes the additional product of cleavage downstream of the correct site. E, cleavage efficiency of pre-P450(SCC) mutants by yeast MPP. The percentages of the mature forms of P450(SCC) relative to the total amounts of the protein are shown.

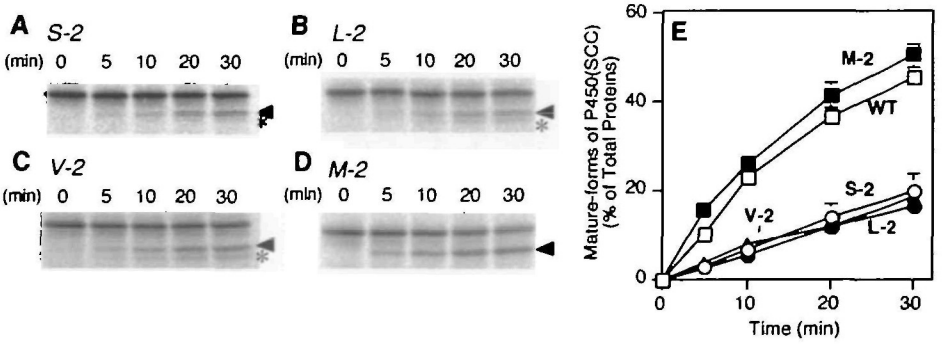

Fig. 3. **Structural limitation of amino acid residue at position -2.** A–D, time courses of cleavage of the S-2, L-2, V-2, and M-2 pre-P450(SCC) mutants. Amino acid sequences of the precursors are represented in Fig. 1. Experimental conditions are as described in the legend of Fig. 2. The arrowheads indicate the mature forms of P450(SCC). The asterisk denotes the additional product of cleavage downstream of the correct site. E, cleavage efficiency of pre-P450(SCC) mutants by yeast MPP. The percentages of the mature forms of P450(SCC) relative to the total amounts of the protein are shown.

by βHis70, βIle180, and βLeu181. Thus, the structural features of the S_2 site also demonstrate the structural requirement of the amino acid at position –2 of the substrate.

Distal Basic Amino Acid Residues of the Presequence Are Required for Entry into the Internal Cavity of MPP—Our earlier studies demonstrated that the distal basic residues around position –10 are important for effective cleavage by MPP (*12–16*). The substitution of His-His-Arg at positions –9 to –11 from the cleavage site of pre-P450(SCC) led to only about a 3-fold reduction in the cleavage rate (Fig. 4A). Previously, a synthetic peptide corresponding to the N-terminal half of pre-P450(SCC) presequence, which contains three basic amino acids, was reported to inhibit strongly the processing activity of MPP, while the peptide corresponding to the C-terminal half, including His-His-Arg, was moderately inhibitory (*23*). The N-terminal three basic residues might, therefore, act as the "distal" ones. These basic amino acids in the N-terminal sequence of the HHR/AAA mutant, Lys-26, Arg-31, and Arg-36 (sequences in Fig. 1), were then successively mutated to alanine. The A-26 mutant resulted in an at least 10-fold decrease in the cleavage rate (Fig. 4B). No processing products of the A-31 and A-36 mutants were observed (Fig. 4, C and D). This result confirms that the distal basic amino acids of the presequence are important for effective cleavage by MPP. We also noted that none of the substitutions of these amino acid led to the additional cleavage product that was observed in the mutants at position –2 (Fig. 4). This indicates that these basic amino acids do not function as determinants of the cleavage site of the presequence. The structure of MPP-pre-COX IV peptide complex showed that the side chains of basic amino acids of the presequence, except that of arginine at position –2, were not involved in interactions with the cavity of the enzyme (*20*). Nevertheless, biochemical studies have to date shown that distal basic residues in the presequences, and acidic ones in the enzyme, are of importance for cleavage reactions (*12–16*). Recently, surface plasmon resonance analysis has demonstrated that ionic strength in the solution affects the association rate between MPP and the substrate (*24*). Taken together with this finding, the distal basic amino acids of the presequence would be required for entry into the negatively charged cavity of MPP.

Structural Elements in the Mature Portion for Cleavage by MPP—Bulky and hydrophobic amino acid residues are frequently found at position +1 from the MPP cleavage site, particularly in R-10 type precursors (*11*). The A+1 mutant of pre-P450(SCC), which has alanine substituted for isoleucine, showed a drastic decrease in the cleavage rate (Fig. 5A). S+1 and G+1 mutants were no longer cleaved at the original site, but were slightly cleaved at the downstream site (Fig. 5, B and C). On the other hand, the cleavage rate of the F+1 mutant increased by at least 3-fold compared to the wild type (Fig. 5D). In a cleavage analysis of the fluorogenic peptide modeled on rat malate dehydrogenase precursor (pre-MDH), in which the amino acid at the position +1 is phenylalanine, the A+1 peptide gave slightly lower cleavage efficiency than the L+1 peptide, whereas the cleavage rates of the G+1 and S+1 peptides were below the detection limit (*16*). Thus, the preferred amino acid at position +1 of pre-P450(SCC) seems to be the almost same as

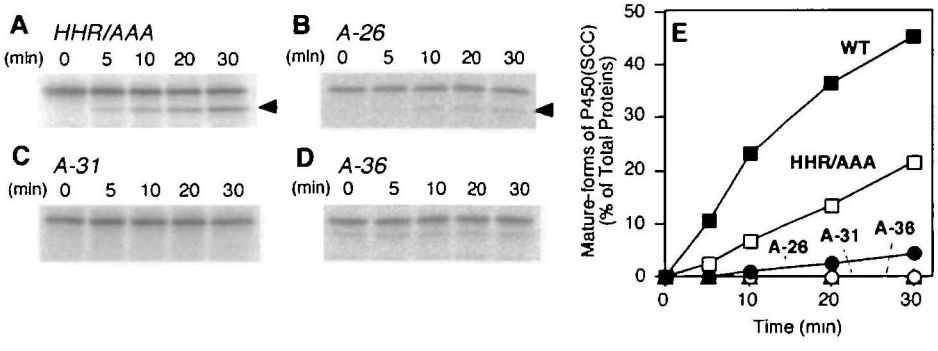

Fig. 4. **Effects of mutations of basic residues distal to the cleavage site on the cleavage rate.** A–D, time courses of cleavage of the HHR/AAA, A-26, A-31, and A-36 pre-P450(SCC) mutants. Amino acid sequences of the precursors are represented in Fig. 1. Experimental conditions are as described in the legend of Fig. 2. The arrowheads indicate the mature forms of P450(SCC). E, cleavage efficiency of pre-P450(SCC) mutants by yeast MPP. The percentages of the mature forms of P450(SCC) relative to the total amounts of the protein are shown.

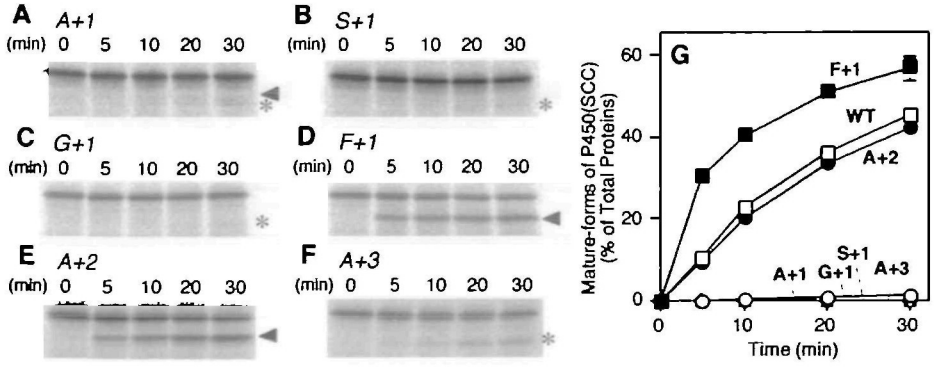

Fig. 5. **Effects of mutations in the mature portion on the cleavage rate.** A–F, time courses of cleavage of the A+1, S+1, G+1, F+1, A+2, and A+3 pre-P450(SCC) mutants. Amino acids sequences of the precursors are represented in Fig. 1. Experimental conditions are as described in the legend of Fig. 2. The arrowheads indicate the mature forms of P450(SCC). The asterisk denotes the additional product cleaved downstream of the correct site. G, cleavage efficiency of pre-P450(SCC) mutants by yeast MPP. The percentages of the mature forms of P450(SCC) relative to the total amounts of the protein are shown.

that for MDH. The structure of yeast MPP-pre-COX IV peptide revealed that βPhe77 provides the S_1' site for the leucine at position +1 (*20*), showing that MPP prefers an aromatic or hydrophobic amino acid at position +1 of the substrate.

According to the conserved cleavage-site motifs proposed by Gavel *et al.* (*11*), serine residues at position +2 in the R-2, R-10, and R-none groups are often found. We have previously demonstrated that a peptide substrate with only proximal and distal arginine residues and phenylalanine at position +1 was not cleaved, though it showed inhibitory effects to MPP activity (*17*). Consequently, it followed that introduction of serine or threonine residues into positions +2 to +4 was required for processing by MPP, and that two or three consecutive serine residues in positions +2 to +4 enhanced the cleavage efficiency (*17*). For pre-P450(SCC), replacement of serine with alanine at position +2 had little effect on the cleavage rate (Fig. 5E), and replacement of threonine at position +3 with alanine abolished cleavage at the authentic site (Fig. 5F). In contrast, serine at position +2 was critical for the recognition of pre-MDH by MPP (*14*). Thus, the requirements of hydrophilic residues at positions +2 and +3 seem to differ with precursors. In the crystal structure, we could not determine the locations of cystein and serine at position +2 and +3 of pre-COX IV, respectively, because of the low electron density in this portion (*20*). In addition, the highly conserved glycine-rich loop of α-MPP also showed a low electron density in the MPP-peptide complex, indicating its flexibility. This loop is closed to the active site of β-MPP in the structure of the uncomplexed MPP. Deletion of this loop led to a significant reduction in affinity for the substrate (*8*). These observations suggest that interaction between the glycine-rich loop of α-MPP and amino acids at positions +2 and +3 of the substrate may be crucial to substrate binding and product release.

Conclusion—In this study, we have demonstrated that alanine at position –2 of pre-P450(SCC) is recognized by MPP as a determinant for processing at the correct site. Because of the tolerance to methionine mutation, this recognition appears to occur through a hydrophobic interaction between the straight methylene side-chain of amino acid at this position and the corresponding subsite. This appears to be a common interaction required for recognition of precursors with arginine at position –2. The mode of pre-P450(SCC) for recognition by MPP is therefore essentially the same as those of R-2 and R-10 precursors, though there is no electrostatic interaction with the guanidino group of arginine in the presequence.

In a survey of the cleavage site in mitochondrial precursors from databases and the literature, there was no common structural feature of amino acids at position –2 of R-none type precursor (*11*). In most mitochondrial precursors, the cleavage sites were determined by the N-terminal sequences of the mature proteins purified from mitochondria. Many R-none precursors might be cleaved by other proteases after MPP processing. If the direct cleavage of these precursors and the N-terminal amino acid sequences are analyzed, it is revealed that the cleavage-sites of mitochondrial presequences are more conserved.

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