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The mitochondrial processing peptidase: function and specificity

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Abstract. Targeting signals of mitochondrial precursors are cleaved in the matrix during or after import by the mitochondrial processing peptidase (MPP). This enzyme consists of two nonidentical α- and β-subunits each of molecular weight of about 50 kDa. In mammals and fungi, MPP is soluble in the matrix, whereas in plants the enzyme is part of the cytochrome bc_1 complex. MPP is a metalloendopeptidase which has been classified as a member of the pitrilysin family on the basis of the $HXXEHX_{76}E$ zinc-binding motif present in β-MPP. Both subunits of MPP are required for processing activity. The α-subunit of MPP, which probably recognizes a three-dimensional motif adopted by the presequence, presents the presequence to β-MPP, which carries the catalytic active site. MPP acts as an endoprotease on chemically synthesized peptides corresponding to mitochondrial presequences. Matrix-targeting signals and MPP cleavage signals seem to be distinct, although the two signals may overlap within a given presequence. The structural element helix-turn-helix, that cleavable presequences adopt in a membrane mimetic environment, may be required for processing but is not sufficient for proteolysis. Binding of the presequence by α-MPP tolerates a high degree of mutations of the presequence. α-MPP may present a degenerated cleavage site motif to β-MPP in an accessible conformation for processing. The conformation of mitochondrial presequences bound to MPP remains largely unknown.

Key words. Mitochondrial import; metallopeptidases; processing; matrix-targeting sequences; zinc-binding motif; holoenzyme.

Introduction

Most nuclear encoded mitochondrial precursors are targeted to mitochondria by amino-terminal presequences [1]. These presequences are cleaved during or after import by a processing enzyme that was initially isolated from the matrix of different organisms [2–8]. The processing activity was associated with a soluble complex with an approximate molecular weight of 100 kDa and was inhibited by metal-chelating agents but not by serine-protease inhibitors. The enzymatic activity was maximal between pH 7 and 8 and was dependent on divalent metal ions such as Mn^{2+}, Zn^{2+} and Co^{2+}. The mitochondrial processing peptidase (MPP) was first purified from *Neurospora crassa* [9] and then from other organisms. MPP consists of two nonidentical α- and β-subunits (α-MPP and β-MPP) of 57 and 52 kDa in *N. crassa* [9], 52 and 50 kDa in *Saccharomyces cerevisiae* [10], and 55 and 50 kDa in rat liver [11, 12]. In *Neurospora*, the β-subunit of MPP has been shown to be identical to the core 1 of the bc_1 complex and turns out to be a bifunctional protein [13]. In plants, MPP is not soluble but resides in the inner mitochondrial membrane [14, 15] as part of a protein complex of the respiratory chain, the cytochrome bc_1 complex [16–18].

The subunits of MPP, which can be viewed as the last components of the import machinery, were the first genetically identified import components. Two temperature-sensitive yeast mutants accumulating precursor proteins were initially isolated by Yaffe and Schatz [19]. The mutants were found to be affected in the essential genes encoding the subunits of MPP [20–23]. The MPP genes were then cloned from rat liver and potato tuber cDNA libraries and proved to be homologous to each other [12, 14, 17, 24, 25]. Studies with the mutants suggested that they accumulated uncleaved precursor proteins outside the mitochondrial inner membrane [19]. It was proposed that β-MPP played a role in the import of precursor proteins. It was then found that cells depleted in either of the two MPP subunits were accumulating precursors inside the mitochondria, suggesting that MPP was involved in cleavage but not in import [26].

MPP recognizes a large number of presequences which do not share a consensus primary sequence. The peptidase recognizes a secondary or tertiary structure which defines the border between presequences and the amino termini of the mature polypeptides [27–29]. The higher-order structure that defines the cleavage site is still unknown. This structure might be generated by the binding of the presequence to MPP.

In this review, we will summarize some of the known properties of MPP. We will then discuss the role of each subunit and the specificity of the enzyme.

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Biochemical properties of MPP

MPP is not inhibited by phenylmethylsulfonyl fluoride and pepstatin, but the α-subunit was shown to be sensitive to sulfhydryl reagents [30]. The processing activity of MPP from rat and fungi requires the presence of divalent metal ions [2]; however, the metal atoms must be weakly bound to the enzyme since they are removed by most of the isolation procedures. Thus, on the basis of the constants governing the association of the peptidase with the metal, MPP is not a metalloprotease. In contrast to MPPs from other organisms, the potato enzyme works without addition of metal ions, probably due to the great stability of the protein complex containing the processing peptidase subunits [24]. As will be discussed below, MPP has been classified as a metallopeptidase of the pitrilysin family on the basis of an inverted HEXXH zinc-binding motif present in β-MPP [31, 32].

The two subunits of the yeast and rat enzymes remain associated during most of the purification steps, in contrast to the corresponding subunits of the *N. crassa* enzyme [9–11]. The association of both subunits of the yeast enzyme is resistant to high a concentration of salts [33]. Notably, the isolated α-MPP subunit is not able to form a dimer [26]. The individual subunits of the potato peptidase need the structural support of the bc_1 complex for processing activity. This complex is very stable, and its processing activity appears to be highly resistant to salt and detergent [24].

The peptidase is able to recognize and cleave a restricted substrate. A chemically synthesized peptide corresponding to the matrix-targeting signal of an authentic mitochondrial precursor plus eight residues of the mature protein is recognized and cleaved by MPP [34]. Substrates of MPP are cleaved by purified MPP but not degraded, suggesting that MPP acts as an endoprotease [11, 34]. The cleaved matrix-targeting peptides are probably degraded in vivo by other as yet unidentified peptidases. The peptide substrates competitively inhibit the processing activity of MPP [34, 35].

A mitochondrial precursor was purified from *Escherichia coli* and used as substrate for the MPP of *N. crassa* [35]. Analysis of the enzymatic activity indicated that MPP followed Michaelis-Menten kinetics of processing and was characterized by an apparent K_m of 1.27 μM and a V_{max} of 0.27 μM/min [35].

Functional cooperation of the MPP subunits

The relative role of each subunit in the catalytic process remains largely unknown. The availability of individual active subunits and sequence comparison of both subunits of MPP from different species with related enzymes has shed some light on their respective roles.

The mature forms of the MPP subunits were produced in *E. coli* and assembled into an enzymatically active peptidase [36]. Both subunits of MPP were shown to be required for processing activity [26, 35–37]. A radiolabelled purified precursor protein bound to the holoenzyme (α, β) in the presence of a chelating agent was shown to be cross-linked to α-MPP [34]. The binding of the precursor to MPP, unlike cleavage, was not prevented by orthophenanthroline, suggesting that binding and cleavage are distinct processes. Very recently, we have shown that a derivatized purified precursor carrying a photoactivatable group attached to its presequence was cross-linked to α-MPP in the absence of any β-MPP [33]. From all these experiments, it is believed that α-MPP is involved in the binding of the substrate.

On the basis of an alignment of the sequences of the mitochondrial processing enzymes, the *E. coli* protease III (pitrilysin) and three members of the insulin-degrading enzyme family, a superfamily of metalloendopeptidases containing an HXXEH motif was defined [31]. This motif is also found in other peptidases from rat, *Klebsiella*, and *Bacillus subtilis* [38, 39, reviewed in 40]. The HXXEH motif was proposed to be an inversion of the consensus active site HEXXH sequence found in many metalloproteinases [41, 42]. From the three-dimensional structure of the bacterial thermolysin, the two histidines of the HEXXH sequence were proposed to serve as ligands to the zinc, while the glutamic acid was believed to polarize a zinc-bound water molecule for nucleophilic attack on the scissile bond of the substrate. The fourth zinc ligand in thermolysin was found 20 residues downstream of the HEXXH motif [43]. The structures of many proteinases that share the HEXXH motif or even the elongated zinc-binding motif have been now resolved; they provide a detailed understanding of the catalytic mechanisms of these metalloproteinases [reviewed in 44].

An alignment of the sequences of pitrilysin, the human and *Drosophila* insulin-degrading enzymes and β-MPPs, identified two glutamates outside the active site that are conserved in all these proteins [45]. Studies on pitrilysin and on insulin-degrading enzyme indicated that a glutamic acid 76 residues downstream of the HXXEH motif was involved in zinc binding and that the glutamic acid of the conserved motif was the catalytic residue [42, 45, 46]. The new motif, $HXXEHX_{76}E$, was used to define a new family of metallopeptidases: the pitrilysin family [32].

The histidines and glutamic acid of the HXXEH motif from the β-subunit of rat MPP were mutated individually. All three mutants lost their processing activity, indicating that this motif is involved in the formation of the active site of β-MPP [47]. Pitrilysin and the insulin-degrading enzymes have been shown to have an absolute requirement for metal cofactors [48–50]. Published methods for the purification of MPP from fungi and

mammals lead to the loss of the endogeneous divalent cation. The activity of MPP is restored by divalent cations added to the cleavage buffer [9–11]. It has been suggested that the metal requirement for the processing activity of yeast MPP was not absolute when the enzyme was assayed with a 10-fold molar excess of the precursor protein [34]. We have now purified a 6 × His-tagged yeast MPP produced in *E. coli*, using a nickel-agarose resin. Under these conditions, the enzyme retains 50% of its processing activity in the absence of any added divalent cations. Preliminary results indicate that the enzyme purified from *E. coli* contains 0.5 atom of zinc per molecule of holoenzyme. If the purification was continued with an anion-exchange column, most of the metal was lost [33]. It may be possible that the cleavage activity of yeast MPP, assayed with a molar excess of the precursor protein and in the absence of added metal cofactor, is accounted for by residual holoprotease containing divalent cations.

Except for the *N*-arginine dibasic convertase which cleaves peptide substrates on the N-terminus of Arg residues in dibasic stretches [39], peptidases from the pitrilysin family recognize a three-dimensional structure rather than an amino acid sequence. The α-subunit of MPP probably recognizes a structural motif of the mitochondrial presequences and presents it to the β-subunit, which carries the catalytic active site. The conserved glycine-rich region of the α-MPPs [17], which is not found in any β-MPPs or in other members of the pitrilysin family, might be a crucial region for binding of the peptide substrate. In order to identify important regions in β-MPP involved in processing, outside the active site HXXEH, we have introduced alanine insertions all along the sequence of yeast β-MPP. We then analysed the effect of the mutations on processing activity and on the association of the subunits with each other. Alanine insertions after Ser-114 and Ser-314 prevent processing activity without affecting the interaction between the subunits [33]. The enzymatic cleavage may depend on the correct positioning of the substrate by α-MPP in some key regions of β-MPP. These regions may lie around Ser-114 and Ser-314. We also found that the region containing Lys-215 may be involved in the association with the α-subunit. The determination of the three-dimensional structure of MPP or of its subunits will provide a more detailed understanding of the catalytic mechanism and of the functional cooperation between both subunits.

Specificity of MPP

In addition to the information required for recognition and processing by MPP, mitochondrial presequences contain instructions to target precursor proteins to mitochondria and to transport them across the two mitochondrial membranes. The sequence requirement for import seems to be rather nonspecific, since 25% of randomly generated presequences function as import signals [51]. The targeting function does not depend on specific amino acid sequences but rather on a correct overall amino acid sequence [52, 53]. However, most of the randomly generated presequences, as well as the functional artificial matrix-targeting presequences, are not cleaved by MPP. The instructions for import and for cleavage seem to be distinct from one another; nevertheless, structural elements of the presequences recognized by the import apparatus and by MPP may overlap [28].

Mitochondrial presequences are generally enriched in positively charged, hydroxylated and apolar amino acid residues, and they appear to form positively charged amphiphilic structures that are usually α-helical [53–55]. Arginines are preferentially found at positions -2, -3 or -10 relative to the cleavage site [56]. Arginine at position -2 of precytochrome b_2 and at position -2, -3 or -10 of premalate dehydrogenase were shown to be necessary for cleavage [35, 57].

Two-dimensional proton NMR, combined with circular dichroism, has enabled the conformation of several mitochondrial presequences to be determined. Mitochondrial presequences are essentially unstructured in water but tend to adopt an α-helical conformation in a membrane mimetic environment. In the presence of micelles of phospholipid analogs, the presequence of the subunit IV of the cytochrome oxidase (p25) has an amphiphilic structure at its N-terminus followed by a less structured C-terminal region [58]. Upon addition of cardiolipin (an anionic phospholipid) or in a negatively charged membrane mimetic environment, the micelle-bound p25 contains two helices separated by a proline residue at position 13 [59, 60]. The mitochondrial presequence derived from the F_1-ATPase β-subunit also has two helical domains, the N-terminal helix being somewhat more stable [61]. Similarly, the presequence of rat liver aldehyde dehydrogenase (pALDH) contains two amphiphilic α-helices, one located at the N-terminus and the other located at the C-terminus, both separated by an Arg-Gly-Pro flexible linker region [62]. Switching the positions of the C- and the N-helices of pALDH did not prevent processing, although it occurred at a lower rate [63]. Furthermore, the break introduced by a proline residue and the second, less structured helix appear to be important for MPP recognition [57, 64–68]. The structures of the N-terminal sequences of rhodanese and 3-oxoacyl-CoA thiolase, two mitochondrial presequences that are not processed by MPP, have been determined [65]. Interestingly, the authors found that these uncleaved presequences form a long α-helix beginning near the N-terminus and extending for at least three helical turns. This result, together with the finding that a mutant of pALDH with its flexible linker region

deleted was not processed by MPP and formed a continuous α-helix [64], led to the proposal that a mitochondrial presequence with an N-terminal α-helix longer than 11 residues cannot adopt the conformation needed for processing [65]. This hypothesis is supported by the observation that mutations of a precursor protein many residues upstream or downstream from the cleavage site can inhibit cleavage by MPP [27, 28]. However, Jarvis et al. [68] found that the mitochondrial matrix chaperonin 10 contained a nonprocessed presequence with a helix-turn-helix motif comparable to those of the processed presequences described above. This result indicates that a flexible region separating two α-helical regions may be required for recognition by MPP, but it is not sufficient for processing [68]. Klaus et al. [69] proposed that N- and C-terminal regions flanking a degenerated sequence motif around the scissile bond are not required for the specificity of MPP cleavage but contribute to the efficiency of processing. The specificity of processing could be achieved by presenting to MPP a degenerated cleavage site motif in an accessible conformation. Analysis of the conformation of various unprocessed and processed mutants of a given cleavable presequence peptide will be required to define clearly the conformation of presequences recognized and cleaved by MPP.

The question arises whether the conformation of the presequence peptides observed in the presence of detergents is the conformation recognized by MPP, since highly purified MPP cleaves p25 in water in the absence of any detergent or lipids [34]. One remaining challenge would be to determine the conformation of peptide substrate bound to MPP.

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