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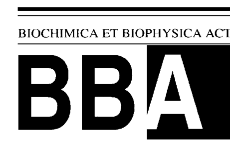

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Review

Mitochondrial processing peptidases

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Abstract

Three peptidases are responsible for the proteolytic processing of both nuclearly and mitochondrially encoded precursor polypeptides targeted to the various subcompartments of the mitochondria. Mitochondrial processing peptidase (MPP) cleaves the vast majority of mitochondrial proteins, while inner membrane peptidase (IMP) and mitochondrial intermediate peptidase (MIP) process specific subsets of precursor polypeptides. All three enzymes are structurally and functionally conserved across species, and their human homologues begin to be recognized as potential players in mitochondrial disease.
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Keywords: Mitochondrion; Protein import; Processing peptidase; Presequence

1. Introduction

Mitochondria can only originate from the growth and division of preexisting mitochondria. This process depends on a continuous supply of proteins, only a few of which are encoded by the mitochondrial genome and translated within mitochondria. The vast majority of mitochondrial proteins (~ 500 in *Saccharomyces cerevisiae* and ~ 1000 in mammals) are encoded by nuclear genes, translated on cytosolic ribosomes, and posttranslationally transported to the mitochondria. Most proteins destined for the mitochondrial matrix and many of those targeted to the intermembrane space and the inner membrane are initially synthesized as larger precursor polypeptides carrying N-terminal extensions that are cleaved off by specific processing peptidases once each precursor has reached its final destination within the mitochondrion. Most outer membrane proteins and carrier proteins of the inner membrane are otherwise synthesized without cleavable presequences and instead carry N-terminal, C-terminal and/or internal signal sequences. There are a number of excellent reviews on mitochondrial protein import (e.g. other reviews in this issue), and the main steps and molecular components involved in this process are only briefly summarized below.

Cleavable presequences normally consist of a positively charged matrix-targeting signal of ~ 20–60 residues, often followed by an additional intramitochondrial sorting signal [1]. Presequences are both necessary and sufficient to correctly target the mature protein (i.e. the form found in mitochondria at steady state) to its final destination within the mitochondrion. In fact, presequences have been successfully used to target nonmitochondrial passenger molecules to the organelle, including cytoplasmic proteins and even nucleic acids [2,3]. In the cytoplasm, the presequence is required for recognition of the nascent precursor polypeptide by protein chaperons that prevent aggregation and degradation of the precursor and maintain it in an "import-competent" conformation [4–7]. Import is initiated by interaction of the presequence with the translocase of the outer membrane or TOM complex, with translocation across the outer membrane being driven by electrostatic interactions between the positively charged presequence and acidic domains of the Tom22 and Tom40 proteins [8]. Translocation across the inner mitochondrial membrane requires the electrochemical potential ($\Delta\Psi$) [9,10], the translocase of the inner membrane or TIM complex, and mitochondrial Hsp70 on the matrix side of the inner membrane [11–13], the motor that pulls the precursor across both membranes at the expense of ATP hydrolysis [14]. Once the precursor has been translocated, the matrix-targeting signal is no longer necessary and may actually interfere with further sorting and/or protein folding and assembly. Mitochondrial process-

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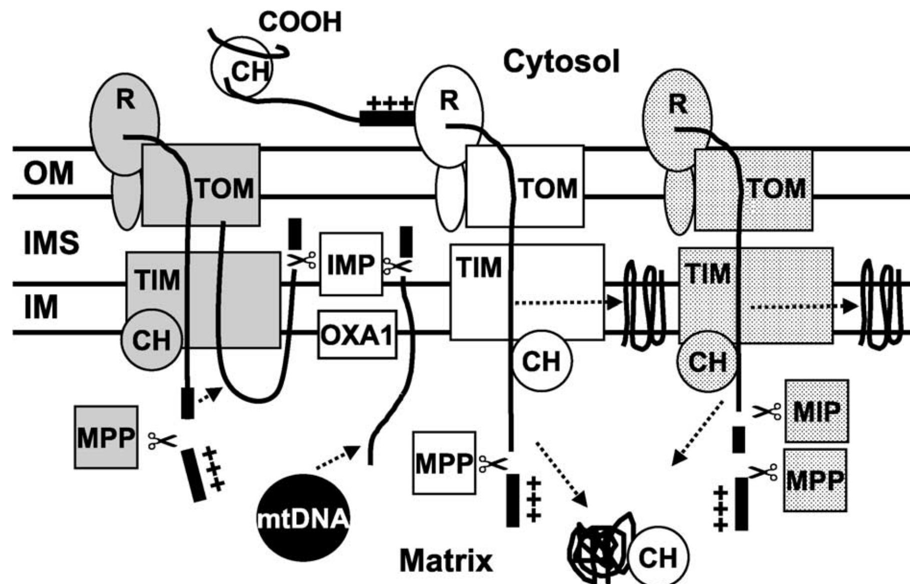

Fig. 1. Proteolytic processing of mitochondrial protein precursors. CH, cytoplasmic and mitochondrial chaperones; R, outer membrane receptors associated with TOM, the translocase of the outer membrane; TIM, translocase of the inner membrane; OM, outer membrane; IMS, inter membrane space; IM, inner membrane; MPP, mitochondrial processing peptidase; MIP, mitochondrial intermediate peptidase; IMP, inner membrane peptidase; mtDNA, mitochondrial DNA; OXA1, OXA1 complex. See text for details.

ing peptidase (MPP; EC 3.4.24.64) takes care of removing the matrix-targeting presequence from most precursors (Fig. 1). This enzyme processes precursors that are fully translocated to the matrix as well as precursors in transit to the inner membrane or the intermembrane space. Many of the precursors destined for the intermembrane space have bipartite presequences consisting of a matrix-targeting signal followed by an intermembrane space-sorting signal. The matrix-targeting signal is cleaved by MPP, while the intermembrane space-sorting signal is removed by inner membrane peptidase (IMP; EC 3.4.99) in the inner membrane (Fig. 1). A single cleavage by MPP is normally sufficient for the maturation of most matrix and inner membrane protein precursors, with the exception of the octapeptide-containing precursors, that require two cleavages, sequentially carried out by MPP and mitochondrial intermediate peptidase (MIP; EC 3.4.24.59), also localized to the matrix (Fig. 1).

2. Mitochondrial processing peptidase

2.1. Nomenclature

MPP was initially purified from different organisms by independent laboratories that assigned different names to each of the enzyme's two subunits. The names MPP, MPP-1, MAS2, MIF2, P-55 and cytochrome *c* reductase subunit III have been used in the past for α-MPP, and the names PEP, MAS1, MIF1, P-52 and cytochrome *c* reductase subunit I and II for β-MPP [15]. Based on a unified nomenclature established in 1993, the larger subunit is currently designated α-MPP, and the smaller subunit β-MPP [15]. MPP from plants is one exception to this general rule as here the enzyme is integrated into the cythochrome bc_1 complex. In different plants, this complex contains two to four Core proteins that are identical to α-MPP (Core 2/α-MPP subunits) or β-MPP (Core 1/β-MPP subunits) (reviewed in Ref. [16]).

2.2. Cleavage specificity

Initial sequence analyses of mitochondrial precursor proteins revealed three main features found in the majority of presequences cleaved by MPP across eukaryotes, namely, (i) an overall positive charge, (ii) a predicted ability to form an amphiphilic α-helix [1,17,18], and (iii) the presence of an arginine residue at the −2 position (the "R-2 rule") from the cleavage site [1,19]. Four cleavage site motifs were later identified [20]: xRx↓x(S/x) (R-2 motif); xRx(Y/x)↓(S/A/x)x (R-3 motif); xRx↓(F/L/I)xx(S/T/G)xxxx↓ (R-10 motif, which is cleaved sequentially by MPP and MIP; the arginine residue is at position −2 from the MPP cleavage site and −10 from the MIP cleavage site); xx↓x(S/x) (R-none motif) (Fig. 2). In a survey of 71 mitochondrial matrix-targeting presequences from *S. cerevisiae*, R-2, R-3 or R-10 motifs were found in only ∼65% of the presequences [21], and similar results were obtained in a survey of plant mitochondrial presequences [22]. Thus, there is a relatively high degree of amino acid sequence degeneracy at MPP cleavage sites, and this limits the level of confidence with which such sites can be predicted by statistical or other methods [19,20,23]. These predictions are often complicated by the fact that following an initial cleavage by MPP, many presequences are cleaved again by either MIP or IMP, with

R-2 or R-3 Motif

```
                                                          ↓MPP
HMUT     (A59145)       MLRAKNQLFLLSPHYLRQVKESSGSRLIQQRL  LHQQ..
HPDHE3   (P09622)    MQSWSRVYCSLAKRGHFNRISHGLQGLSAVPLRTY  ADQP..
MAAT     (J02622)          MALLHSGRVLSGVASAFHPGLAAAASARA  SSWW..
RSCSA    (J03621)            MVSGSSGLAAARLLSRTFLLQQNGIRH  GSYT..
YCOX8    (P04039)            MLCQQMIRTTAKRSSNIMTRPIIMKRS  VHFK..
YDHA7    (P46367)               MFSRSTLCLKTSASSIGRLQLRYF  SHLP..
YIDH1    (P28834)                           MLNRTIAKRTL  ATAA..
YRM02    (P12687)            MWNPILLDTSSFSFQKHVSGVFLQVRN  ATKR..
```

R-10 Motif

```
                                                                    ↓MIP
HOTC     (K02100)                   MLFNLRILLNNAAFRNGHNFMVRN  FRCGQPLQ  NKVQ..
MMDH     (M16229)                           MLSALARPVGAALRRS  FSTSAQNN  AKVA..
NCPB     (P10255)   MFGPRHFSVLKTTGSLVSSTFSSSLKPTATFSCARA  FSQTSSIM  SKVF..
YAT14    (Q12349)                   MFPIASRRILLNASVLPLRLCNRN  FTTTRISY  NVIQ..
YCOXIV   (P04037)                          MLSLRQSIRFFKPATRT  LCSSRYLL  QQKP..
YDLDH    (P09624)                              MLRIRSLLNNKRA  FSSTVRTL  TINK..
YFe/S    (P08067)                  MLGIRSSVKTCFKPMSLTSKRL  ISQSLLAS  KSTY..
YRM07    (P36519)                                MQRFSLVTHRS  FSHSCVKP  KSAC..
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No R-2, R-3 or R-10 Motif

```
BMPC     (X05340)  ..NAPHLQLVHDGLAGPRSDPAGPPGPPRRSRNLAAA  AVEE..
HF0A     (M16453)  ..GALRRLTPSAALPPAQLLLRAVRRRSHPVRDYAAQ  TSPS..
NATP2    (P17614)  ..GLISRSLGNSIPKSASRASSRASPKGFLLNRAVQY  ATSA..
PCS      (P00889)            MALLTAAARLFGAKNASCLVLAARHAS  ASST..
YATP6    (P00854)                            MFNLLNTYIT  SPLD..
YADH3    (P07246)            MLRTSTLFTQRVQPSLFSRNILRLQST  AAIP..
YDHA1    (P22281)                  MLATRNLVPIIRASIKWRIKL  SALH..
YNDI1    (P32340)             MLSKNLYSNKRLLTSTNTLVRFASTR  STGV..
YRM04    (P36517)                        MWKRSFHSQGGPLR  ARTK..
```

Fig. 2. Motifs found at the MPP and MIP cleavage sites of mitochondrial protein presequences. Symbols and GenBank accession numbers are given in the figure. BMPC—bovine mitochondrial phosphate carrier; HF0A—human F_0-ATPase proteolipid subunit; HMUT—human methylmalonyl-CoA mutase; HOTC—human ornithine transcarbamylase; HPDHE3—human pyruvate dehydrogenase E3 subunit; MAAT—mouse aspartate amino tranferase; MMDH—mouse malate dehydrogenase; NATP2—*Nicotiana plumboginifolia* ATP synthase beta chain; NCPB—*N. crassa* cyclosporin-binding protein; PCS—pig citrate synthase; RSCSA—rat succinyl-CoA synthetase α subunit; YADH3—*S. cerevisiae* alcohol dehydrogenase III; YAT14—*S. cerevisiae* ATP synthase H chain; YATP6—*S. cerevisiae* ATP synthase A chain; YCOX4—*S. cerevisiae* cytochrome *c* oxidase subunit IV; YCOX8—*S. cerevisiae* cytochrome *c* oxidase subunit VIII; YDHA1—*S. cerevisiae* aldehyde dehydrogenase; YDHA7—*S. cerevisiae* potassium-activated aldehyde dehydrogenase; YDLDH—*S. cerevisiae* dihydrolipoamide dehydrogenase; YFe/S—*S. cerevisiae* ubiquinol–cytochrome *c* reductase Rieske iron–sulfur protein; YIDH1—*S. cerevisiae* isocitrate dehydrogenase subunit 1; YNDI1—*S. cerevisiae* rotenone-insensitive NADH-ubiquinone.

each of these three enzymes possessing different requirements for substrate recognition.

A large body of mutational studies has shown that the R-2 or R-3 motif is important but not sufficient to direct cleavage by MPP. Site-directed mutagenesis of the −2 or −3 arginine in different precursor molecules has had variable results, including complete or partial inhibition of processing, creation of novel cleavage sites or apparent lack of any obvious effect [24–30]. The role of other positively charged residues in the presequence is even less defined. Mild to severe effects on processing by MPP have been noted even when some or all of the arginine and lysine residues were replaced in different precursors [25,27,31–33], whereas a number of replacements of non-basic amino acids had no significant effect on the processing of either natural precursors or synthetic leader peptides [31,34,35]. Further complicating this picture, processing by MPP can also be affected by small deletions or point mutations within the 8–10 amino acids C-terminal to the MPP cleavage site [36–38], and even by point mutations in the mature portion of the precursor far away from the cleavage site [39,40]. Collectively, these findings have led to the conclusion that structural elements in the presequence, and probably the mature portion as well, represent the most important determinants for cleavage of any given precursor by MPP. Accordingly, NMR and circular dichroism studies have shown that synthetic presequences form specific helix–linker–helix structures in solution, and it has been suggested that this may be the conformation recognized by MPP [41,42]. However, the recently resolved crystal structures of two synthetic presequences co-crystallized with yeast MPP has revealed each presequence bound in an extended conformation [43]. The authors have proposed that the conformation of the signal sequence is context-dependent, i.e. the presequence would be in an amphiphilic α-helical conformation when it interacts with TOM and TIM components in the mitochondrial membranes, and in an extended conformation when it interacts with MPP in the matrix [43]. One caveat is that the two peptide substrates used in this study correspond to the presequences of the yeast malate dehydrogenase and the yeast cytochrome *c* oxidase subunit 4, two octapeptide-

containing precursors that are processed in two sequential steps by MPP and MIP [21]. As we will see later, the presequences of these precursors possess features that allow them to be cleaved by two peptidases, with the two cleavages being only eight amino acids apart [19,36,44]. In addition, their MPP cleavage sites are characterized by the presence of an arginine at -2 and a phenylalanine or a bulky hydrophobic residue at $+1$, features that are typical of the octapeptide-containing precursors but are not necessarily found in precursors cleaved in one step by MPP [19,21] (Fig. 2).

2.3. Biochemical properties

MPP was originally purified from *Neurospora crassa* [45], *S. cerevisiae* [46], and rat liver [47,48], and more recently from potato [49], bovine liver [35], wheat [50], and spinach [51]. MPPs from yeast, *N. crassa* and mammalian sources are soluble heterodimers of 100–110 kDa. Whereas the yeast and rat MPP heterodimers are stable [46,48], the two subunits of *N. crassa* MPP can be separated from each other during purification [45]. The subunits are inactive by themselves, however, and *N. crassa* MPP functions as a heterodimer [28] just like its yeast and mammalian orthologues [52–54]. The association of MPP activity with the cytochrome bc_1 complex in the inner membrane was initially regarded as unique to potato MPP [49], but further studies have shown that this association is probably common in plants (reviewed in Ref. [16]), and that a somewhat similar situation may exist in mammalian mitochondria and possibly other species as well. No MPP activity was detected in crystalline bovine heart mitochondrial cytochrome bc_1 complex, which otherwise possessed full electron transfer activity. When the complex was treated with increasing concentrations of Triton X-100 at 37 °C, however, electron transfer activity decreased and peptidase activity increased. Maximum MPP activity was obtained when the electron transfer activity was completely inactivated [55]. MPP activity in the complex is normally inhibited by the binding of the mitochondrial targeting presequence of the Rieske protein that is lodged between the two Core subunits at the matrix side of the complex [56] and was probably removed by treatment with Triton X-100 in the study by Deng et al. [55].

Purified MPPs from different sources exhibit optimal activity at pH 7–8 and are inactivated at lower pH. The enzyme is inhibited by EDTA or *o*-phenantroline, and stimulated by divalent metals, mainly Co^{2+} and Mn^{2+} [57–61]. Although an early study did not detect any metal ions associated with the purified enzyme [62], milder purification conditions have later revealed Zn/MPP and Zn/β-MPP molar ratios of 1.05 and 0.86, respectively [63]. When low concentrations of metals are added to the apoenzyme, the activity is restored by $Zn^{2+} > Mn^{2+} > Co^{2+}$ [63]. Similar to other zinc-dependent metallopeptidases [64], however, excess Zn^{2+} inhibits MPP while excess Mn^{2+} or Co^{2+} do not [54,63]. The Triton X-100-activated MPP in the cytochrome bc_1 complex of bovine mitochondria is also completely inhibited by metal ion chelators, and stimulated by divalent cations [55].

2.4. Genes coding for MPP subunits

Complementation of temperature-sensitive mutants deficient in mitochondrial precursor processing (*mas1/mif1* and *mas2/mif2*) first led to the isolation of the two genes encoding the two subunits of yeast MPP. Sequence analysis of the *MAS1/MIF1* and *MAS2/MIF2* alleles showed that they coded for two highly homologous proteins, Mas1p/Mif1p (β-MPP), and Mas2p/Mif2p (α-MPP) [46,65–68]. Two cDNAs coding for the *N. crassa* MPP subunits were isolated shortly thereafter [45,69]. The sequences for the MPP subunits of rat [48,70], potato [49,71], and man [72,73], as well as many other sources have followed (Fig. 3). All MPP subunits identified to date are synthesized as larger precursor proteins with matrix-targeting presequences, and therefore require the presence of active MPP in the mitochondria in order to be processed to the mature form.

There is ~ 43% identity between yeast and *N. crassa* α-MPP [69] and ~ 36% identity between each of these proteins and the rat α-MPP [48]. For β-MPP, the degree of identity is 45–52% among rat, yeast, and *N. crassa* [45,70]. Finally, there is ~ 20–30% identity between α- and β-MPP subunits within any given species, and slightly lower across species [70,74]. The similarity between MPP subunits and Core proteins of the cytochrome bc_1 complex adds a very interesting twist to this family with three different flavors. In plant mitochondria, MPP is an integral component of the cytochrome bc_1 complex [16,49,51] (although a specific MPP activity has also been detected in the matrix of spinach and soybean mitochondria [75]); the Core 1 and 2 subunits of plant bc_1 complexes share ~ 30–35% identity with β- and α-MPP subunits, respectively, from other organisms [16,49]. In *N. crassa*, the Core 1 protein of the cytochrome bc_1 complex is identical to β-MPP [74]; this bifunctional protein is a component of both the bc_1 complex in the inner membrane and the MPP heterodimer in the matrix [45]. In *S. cerevisiae* and mammals, there are distinct, though related, Core 1 and 2 proteins and α- and β-MPP subunits [70,74,76]. Braun et al. [77] have proposed a model that can explain these findings. Early in evolution, MPP developed starting from a preexisting bacterial protease and became part of the cytochrome bc_1 complex (the situation currently present in plants). In some organisms (e.g. *S. cerevisiae* and mammals), the two MPP subunits later separated from the bc_1 complex (probably through gene duplication events) allowing independent regulation of protein processing and respiration; the *N. crassa* situation would therefore represent an intermediate stage in this process. The primordial MPP subunits (i.e. the Core 1 and 2 proteins) remained an integral part of the bc_1 complex probably because they had become

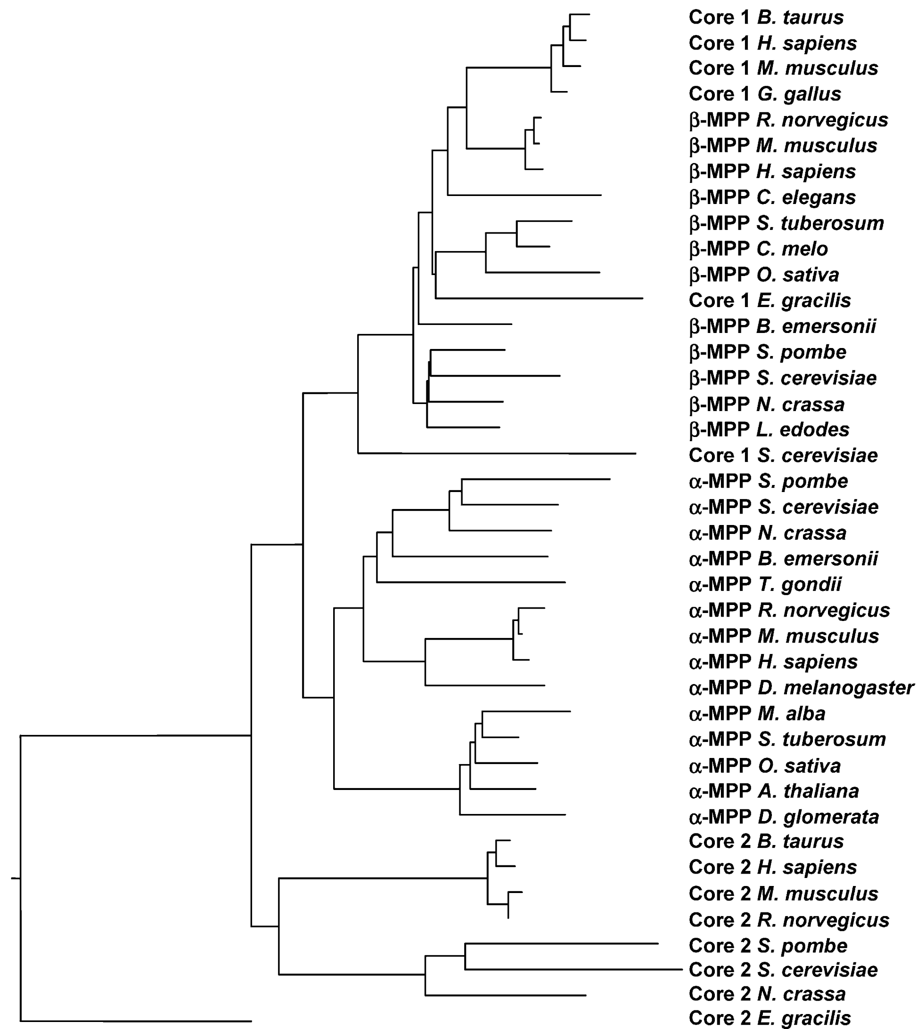

Fig. 3. Phylogenetic tree of α-MPP and β-MPP subunits and Core 1 and 2 proteins of bc_1 complexes from different species. The tree was generated by the MegAlign 4.04 program (Lasergene, DNASTAR). The length of each pair of branches is proportional to the genetic distance between sequence pairs. α-MPP subunits are from: *Homo sapiens* (Q10713); *Rattus norvegicus* (P20069); *Mus musculus* (Q9DC61); *Drosophila melanogaster* (AAF59168); *Morus alba* (BAB62405); *Dactylis glomerata* (AAG42149); *Solanum tuberosum* (P29677); *Arabidopsis thaliana* (AAK59675); *Oryza sativa* (BAB55500); *N. crassa* (P23995); *S. pombe* (CAA22672); *S. cerevisiae* (P11914); *Blastocladiella emersonii* (AAB50243); *Toxoplasma gondii* (AAF00541). β-MPP subunits are from: *H. sapiens* (O75439); *R. norvegicus* (Q03346); *M. musculus* (Q9CXT8); *C. elegans* (CAA92566); *S. tuberosum* (B48529); *O. sativa* (BAB39420); *Cucumis melo* (AAK07827); *N. crassa* (P11913); *Lentinula edodes* (AAD37722); *S. pombe* (CAB66443); *S. cerevisiae* (P10507); *B. emersonii* (AAC63093). The bc_1 complex Core 1 proteins are from: *Bos taurus* (P31800); *H. sapiens* (P31930); *M. musculus* (Q9CZ13); *Gallus gallus* (3659967); *Euglena gracilis* (P43264); *S. cerevisiae* (P07256), and Core 2 proteins from: *B. taurus* (P23004); *H. sapiens* (P22695); *M. musculus* (Q9DB77); *R. norvegicus* (P32551); *S. pombe* (P78761); *S. cerevisiae* (P07257); *N. crassa* (O60044); *E. gracilis* (P43265).

indispensable for its assembly/function. Indeed, the bifunctional nature of the bovine bc_1 complex shows that the MPP/bc_1 complex association found in modern plants represents a situation that was originally common to mitochondria from all organisms [55].

2.5. Structure–function studies

Alignments of β-MPP sequences show two regions of high overall homology within the ~ 200 N-terminal residues of the mature protein. The first is a negatively charged region predicted to form an amphiphilic α-helix with the negatively charged residues clustered on one surface. It was proposed that this domain might recognize positively charged amphiphilic α-helices formed by precursor presequences [48]. The second region is about 70 residues N-terminal to the negatively charged domain and includes an inverted zinc-binding motif, H-x-x-E-H-x_{76}-E, which is present in all known β-MPP subunits (Fig. 4A). This motif is less conserved in Core 1 proteins and α-MPP subunits [70,78] (Fig. 4A). The same motif is found in the insulin-degrading enzyme (IDE) and *Escherichia coli* protease III (pitrilysin), which, together with MPP, have been assigned to the pitrilysin family of zinc-dependent proteases [79]. Replacement of any given residue in the H-x-x-E-H-x_{76}-E motif completely abolishes zinc binding and eliminates

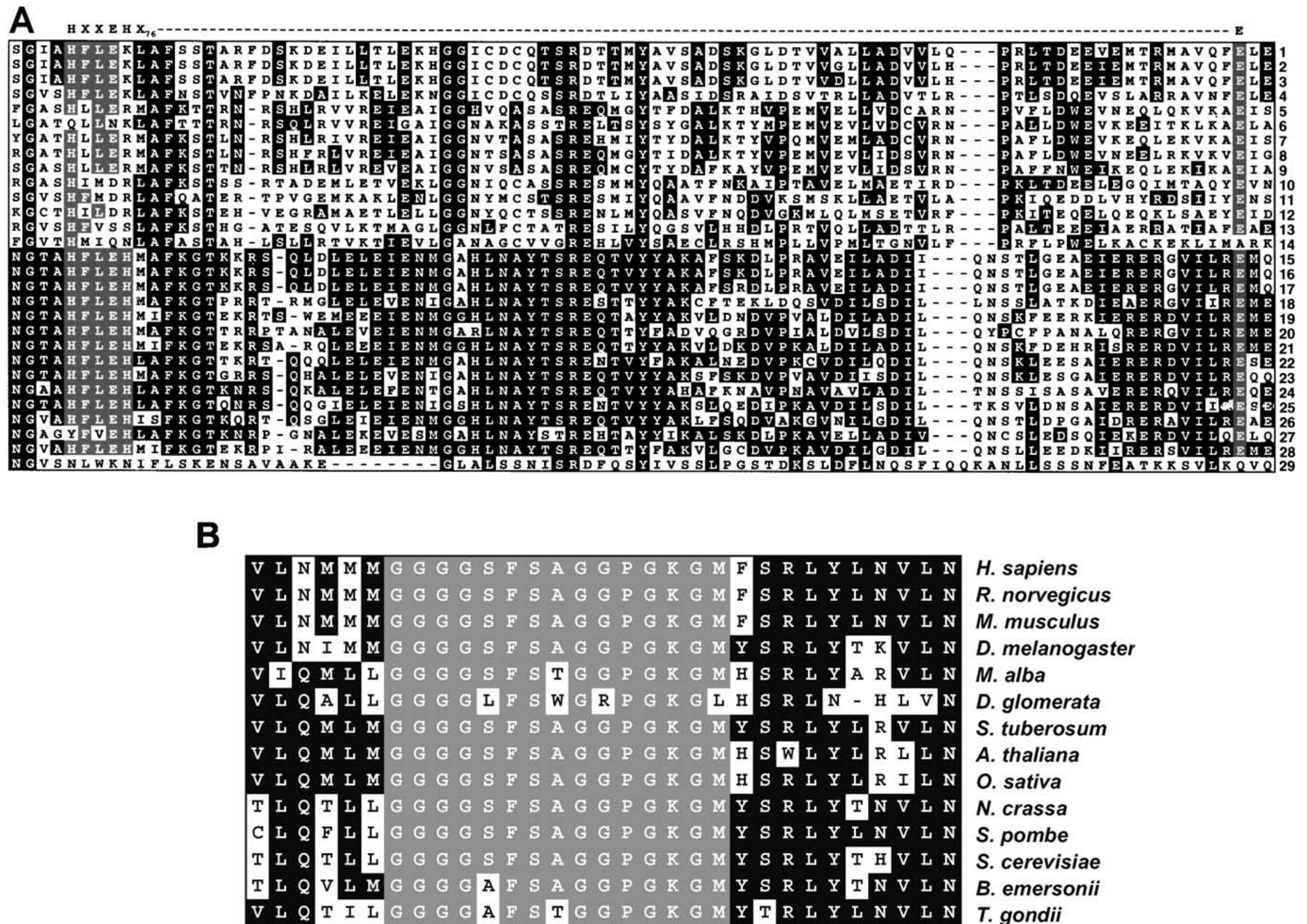

Fig. 4. Conserved functional domains of α-MPP and β-MPP. The alignment was generated by the MegAlign 4.04 program (Lasergene, DNASTAR). (A) Zinc-binding site, and (B) glycine-rich region. Conserved residues are highlighted in black, and the metal binding site and the glycine loop in gray. α-MPP: 1—*H. sapiens* (Q10713); 2—*R. norvegicus* (P20069); 3—*M. musculus* (Q9DC61); 4—*D. melanogaster* (AAF59168); 5—*M. alba* (BAB62405); 6—*D. glomerata* (AAG42149); 7—*S. tuberosum* (P29677); 8—*A. thaliana* (AAK59675); 9—*O. sativa* (BAB55500); 10—*N. crassa* (P23995); 11—*S. pombe* (CAA22672); 12—*S. cerevisiae* (P11914); 13—*B. emersonii* (AAB50243); 14—*T. gondii* (AAF00541). β-MPP: 15—*H. sapiens* (O75439); 16—*R. norvegicus* (Q03346); 17—*M. musculus* (Q9CXT8); 18—*C. elegans* (CAA92566); 19—*S. tuberosum* (B48529); 20—*O. sativa* (BAB39420); 21—*C. melo* (AAK07827); 22—*N. crassa* (P11913); 23—*L. edodes* (AAD37722); 24—*S. pombe* (CAB66443); 25—*S. cerevisiae* (P10507); 26—*B. emersonii* (AAC63093). Core 1 proteins: 27—*B. taurus* (P31800); 28—*S. tuberosum* (AAB28041); 29—*S. cerevisiae* (P07256).

MPP activity [80–82]. These observations have been confirmed by the recently solved crystal structure of yeast MPP, which has shown that the Zn^{2+} ion is coordinated by the two histidine residues and the distant glutamic acid in the H-x-x-E-H-x_{76}-E motif, with the other glutamic acid acting as a general base catalyst on a water molecule that occupies a fourth coordination site on the Zn^{2+} ion [43].

In contrast, mutational and crystallographic analyses have shown that the α-MPP subunit is not directly involved in catalysis [43,80,81,83], but may otherwise be implicated in substrate recognition [43,48,81,84,85]. In particular, a highly conserved glycine-rich segment (Fig. 4B) was initially predicted to form a loop that might act to present the presequence to the active site or to release the cleaved peptide from the enzyme [83]. The crystal structure of the yeast holoenzyme has now revealed that the active site resides in a central, negatively charged cavity between the two subunits, and that the glycine-rich region of α-MPP forms a loop that partially blocks the accessibility of the active site to the substrate [43] (Fig. 5). Another study has shown that deletion of part of this loop results in a significant reduction in both the enzyme's affinity for substrate peptides and the catalytic activity [85]. These data together support a model in which the glycine-rich loop of α-MPP is a flexible element involved in substrate binding and/or product release [43,85].

2.6. Possible roles of MPP in mitochondrial disease

Given the central role played by MPP in the biogenesis of most mitochondrial proteins, a potential involvement of this enzyme in mitochondrial disease is not unexpected. Indeed, recent studies have implicated MPP in the pathophysiology of Friedreich ataxia (FRDA), a relatively

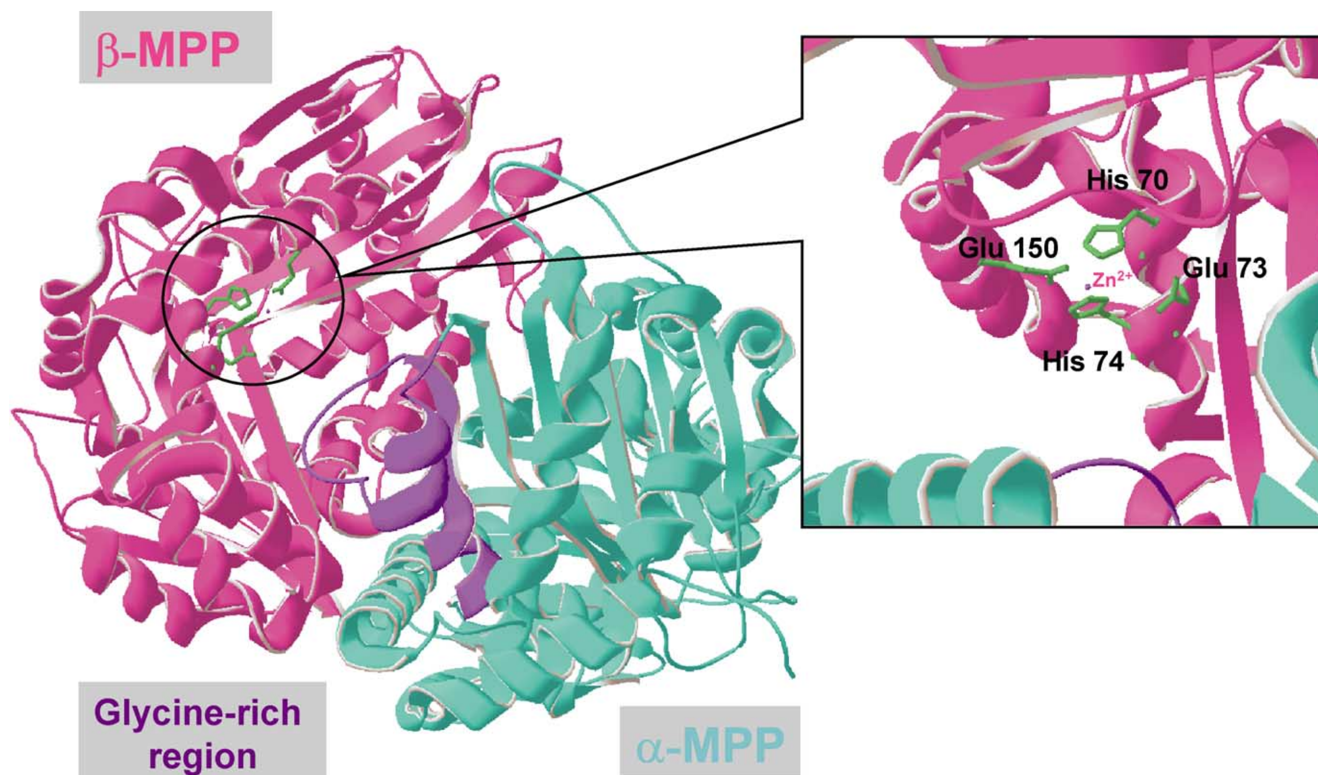

Fig. 5. Structure of yeast MPP (Protein Data Bank accession number: 1HR6). The figure was created using the Swiss-PdbViewer program. The magnified region contains the inverted zinc-binding motif of β-MPP; the glycine-rich loop is colored in purple [43].

frequent (1:50,000 live births) autosomal recessive degenerative disease caused by defects in the mitochondrial protein frataxin (reviewed in Ref. [86]). Similar to most mitochondrial proteins, frataxin is initially synthesized in the cytoplasm as a larger precursor with an N-terminal presequence that targets frataxin to the mitochondrial matrix [87–89]. In a yeast two-hybrid screen, Koutnikova et al. [39] first found a direct interaction between the precursor form of mouse frataxin and β-MPP. It was later shown that both the yeast and the human frataxin precursors are cleaved in two sequential steps, both carried out by MPP [88–92]. This is a rare pattern of processing that has been observed in only one other case, the *N. crassa* ATPase subunit 9 precursor [61]. The presequences of yeast and human frataxin consist of 51 and 55 amino acids, respectively [90,92,93]. The yeast frataxin presequence is cleaved by MPP twice, between residues 20–21 and 51–52, yielding an intermediate and a mature form [88,89,92,93]. Similarly, the human frataxin presequence is cleaved by MPP between residues 41–42 and 55–56 [90]. However, in the case of human frataxin, the precursor is cleaved rapidly and quantitatively to the intermediate form, while the second cleavage is much slower and limits the overall rate at which mature frataxin (which is the form reduced in FRDA patients) is produced [89,90,94].

Processing by MPP is also affected by frataxin point mutations. In a yeast two-hybrid system, interaction between the frataxin precursor and β-MPP was inhibited by two of the most frequent FRDA point mutations, I154F and G130V [39]. The I154F mutation was further found to affect the processing of human frataxin in COS cells [39] but not in vitro [94]. In more recent studies, the W173G mutation inhibited the second processing step both in vitro and in yeast while the G130V mutation had no obvious effect on frataxin processing [90,95]. These observations indicate that processing of frataxin by MPP may be affected by a variety of conditions, including mutations far away from the two cleavage sites and the environment in which processing occurs. The main implication is that there may be a number of genetically determined and/or tissue-specific conditions that reduce (or increase) the rate of frataxin processing by MPP, and thereby influence the severity of FRDA.

3. Inner membrane peptidase

3.1. Substrates

Mitochondrial IMP (EC 3.4.99) catalyzes the maturation of mitochondrial precursor proteins delivered to the intermembrane space (Fig. 1). In *S. cerevisiae*, the enzyme exists as a heterodimeric complex composed of two different subunits: Imp1 and Imp2, both possessing catalytic activity [96–98]. Each subunit is bound to the outer face of the inner mitochondrial membrane through an N-terminal membrane-

spanning domain and projects into the intermembrane space with the C-terminus [99]. Interestingly, each subunit recognizes different substrates. Imp1 is responsible for the maturation of at least three proteins: (i) the mitochondrially encoded cytochrome *c* oxidase subunit 2 (Cox2) [100], (2) the nucleus-encoded cytochrome b_2 precursor (Cyt b_2), which

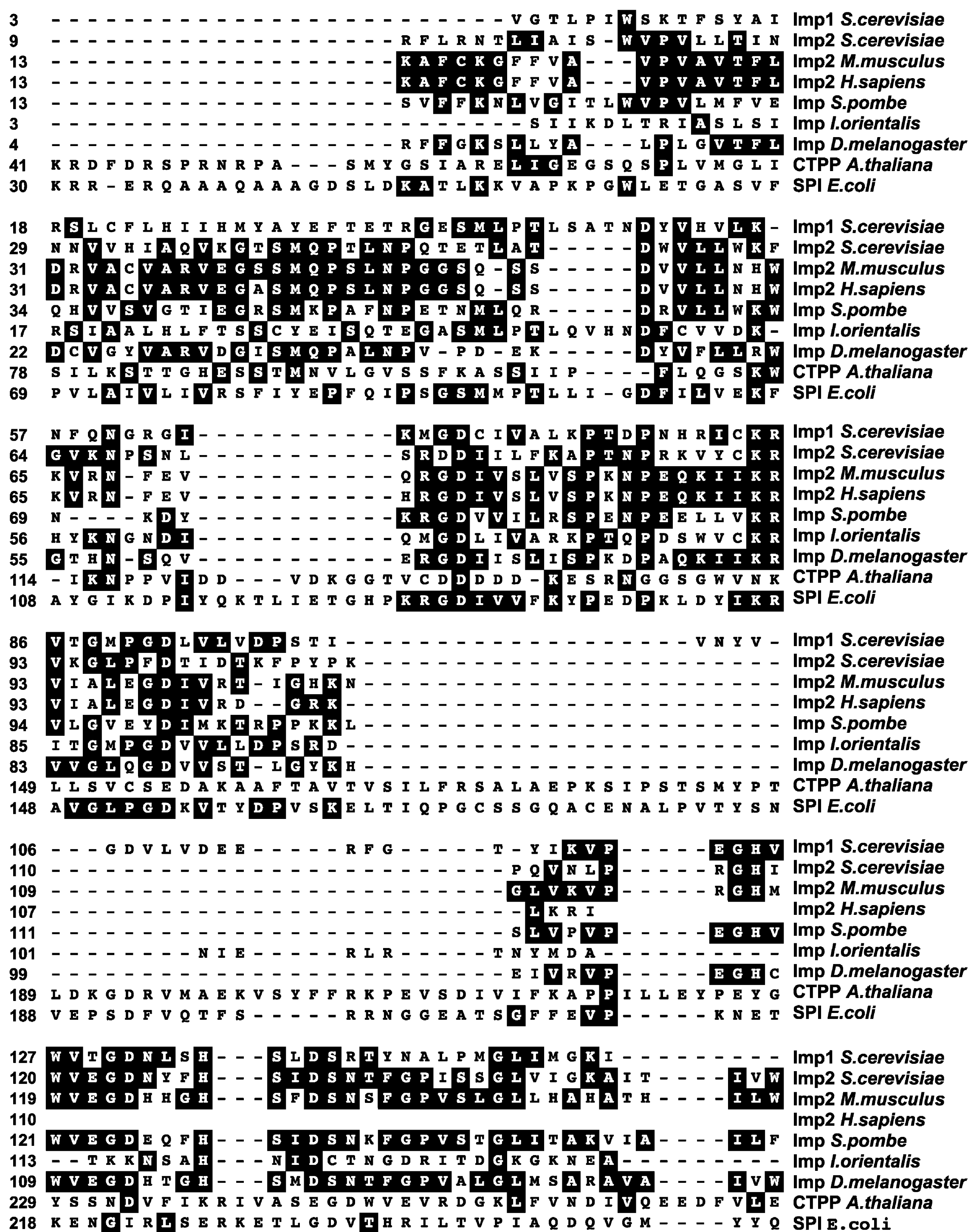

Fig. 6. Comparison of IMP sequences from different species. The alignment was generated using the MegAlign 4.04 program with a gap penalty of 10 and a gap length penalty of 10. Conserved amino acids are highlighted in black. The following sequences are compared: Imp1 (P28627) and Imp2 (P46972) from *S. cerevisiae*; Imp2 from *M. musculus* (AAK52906) and *H. sapiens* (AAH08497); putative IMP subunit from *S. pombe* (T40251), *Issatchenkia orientalis* (AAD11534), and *D. melanogaster* (AAF57438); chloroplast thylakoidal processing peptidase (CTPP) from *A. thaliana* (CAA71502); signal peptidase I (SPI) from *E. coli* (P00803).

is processed sequentially by MPP and Imp1 [101], and (iii) the NADH-cytochrome b_5 reductase (Mcr1), a nucleus-encoded protein of the outer mitochondrial membrane, a soluble form of which is released into the intermembrane space upon cleavage by Imp1 [102]. Imp2 cleaves the nucleus-encoded cytochrome c_1 precursor after an initial cleavage by MPP [96]. Imp2 has been shown to also be necessary for the stability and activity of Imp1 [96,98].

A potential third subunit of IMP, Som1, has been recently described [99]. Based on co-immunoprecipitation and crosslinking experiments, Jan et al. [99] have shown that there exists a direct physical interaction between Imp1 and Som1. They concluded that this interaction is a prerequisite for correct Imp1 peptidase function, and that Som1 may represent an additional subunit of IMP. Som1 is necessary for the cleavage of two of the three known substrates of Imp1 (Cox2 and Mcr1), but is not essential for the maturation of Cyt b_2. Reduced amounts of mature Cyt b_2 are detected in *som1* mutants, suggesting that Imp1 can be at least partially active in the absence of Som1, and that Som1 may normally play a role in substrate recognition [99,103].

3.2. Genes coding for IMP subunits

The *S. cerevisiae IMP1* and *IMP2* genes were identified by complementation of the respiratory-deficient phenotype of yeast strains with defective processing of inner membrane protein precursors [96,104,105]. The enzyme has been purified from yeast mitochondria [98], and found to consist of at least two polypeptides of 21.4 and 19 kDa, respectively. N-terminal sequencing of the two subunits revealed that they were the product of the *IMP1* and *IMP2* genes, respectively [96,105]. The *SOM1* gene was initially identified as a high-copy suppressor of a *imp1* mutant [103].

3.3. Homology to type I leader peptidases

Imp1 and Imp2 share ~ 31% identity and both are ~ 25% identical to the *E. coli* leader peptidase, which removes the pre-sequence of proteins translocated across the bacterial membrane [96,106] (Fig. 6). Both IMP and the *E. coli* leader peptidase are membrane associated and require acidic phospholipids for activity [105,107–109]. In both enzymes, the catalytic domain resides in the C-terminal region and is characterized by highly conserved serine/lysine dyads. Mutational studies have shown that these residues are required for the catalytic activity of both Imp1 and Imp2 [110]. IMP belongs to type I leader peptidases, a group of membrane-bound enzymes including the leader peptidase of the bacterial plasma membrane and the signal peptidases of the endoplasmic reticulum and the thylakoid membrane of chloroplast [105,111–113]. On the other hand, the Som1p sequence does not show any significant homology with the Imp1 and Imp2 sequences, nor with other known proteins.

3.4. Cleavage specificity

The only known natural protein substrate cleaved by Imp2 has an alanine residue at − 1 and a serine residue at − 3 [96], suggesting that the substrate specificity of Imp2 is similar to that of other type I leader peptidases that prefer small uncharged amino acids at those positions [114,115]. Imp1 substrates, however, have an asparagine residue at position − 1 and thus do not conform to the standard motif recognized by type I leader peptidases [104]. Site-directed mutagenesis of the intermediate form of Cyt b_2 (i.e. the form processed by IMP after an initial cleavage of the precursor by MPP) has revealed that the − 1 asparagine is not responsible for the nonoverlapping substrate specificity of Imp1 and Imp2. When different amino acids were substituted for this particular residue, many of the resultant leader peptides were efficiently processed by Imp1 and not by Imp2 [110]. These findings suggest that other determinants, possibly the mature portions of IMP substrates, are responsible for their recognition by either Imp1 or Imp2 [110]. This view is consistent with the early proposal that during evolution, IMP has acquired multiple subunits through gene duplication events, which allowed for a broader substrate specificity [96].

3.5. A human IMP homologue potentially involved in disease

A human gene encoding a potential homologue of yeast Imp2 has been recently reported (inner mitochondrial membrane peptidase 2-like, *IMMP2L*) [116]. The encoded protein shows 31% homology to Imp2, and based on the hydropathy profile, contains one predicted N-terminal transmembrane domain [116]. The *IMMP2L* gene was identified by positional cloning and was found to be disrupted by a duplication and inversion of the 7q31 region associated with Tourette syndrome, a neuropsychiatric disorder [116]. Respiratory chain defects have been associated with many neurodegenerative disorders and are also believed to be implicated in neuropsychiatric disorders [117]. Thus, it has been proposed that *IMMP2L* may be important for the biogenesis of complex III and/or complex IV, and may therefore represent a candidate locus for the etiology of Tourette syndrome and possibly other neuropsychiatric disorders [116].

4. Mitochondrial intermediate peptidase

4.1. Nomenclature

MIP (EC 3.4.24.59) was first characterized in rat liver mitochondria and called P_2 [118] or MPP-2 [48]. It was later designated MIP to indicate that this enzyme cleaves *intermediate-size* proteins initially processed by MPP [36]. The

symbols RMIP, YMIP, SMIP, and HMIP have been used to designate the enzyme from rat [119], *S. cerevisiae* [120], *Schizophillum commune* [121] and man [122]. The gene encoding YMIP, initially designated *MIP1*, was later redesignated *OCT1* [123], and the gene encoding HMIP has been designated *MIPEP* [122].

4.2. Two-step processing by MPP and MIP

Many mitochondrial protein precursors targeted to the mitochondrial matrix or the inner membrane are processed in two sequential steps by MPP and MIP (Fig. 1). These precursors are characterized by the motif R-X↓(F/L/I)-X-X-(T/S/G)-X-X-X-X↓ at the C-terminus of the leader peptide (R-10 motif; see Section 2.2 above) [19,20] (Fig. 2). MPP first cleaves the motif two peptide bonds from the arginine residue, yielding a processing intermediate with a typical N-terminal octapeptide that is sequentially cleaved by MIP yielding mature-size protein (Fig. 2). The octapeptide is required for the initial cleavage by MPP, suggesting that its function may be to provide an MPP-compatible cleavage site in a subset of mitochondrial precursors [36]. Gavel and von Heijne [20] found that the mature N-termini of octapeptide-containing precursors possess more positively and less negatively charged amino acids than the mature N-termini of precursors cleaved in one step by MPP, and suggested that the octapeptide provides a spacer to separate the MPP cleavage site from a positively charged structure that would otherwise interfere with processing. Later studies, however, have shown that the presence of a cleavable octapeptide is not an absolute requirement, at least in the case of the Rieske protein of the cytochrome bc_1 complex [29,124,125].

4.3. Cleavage specificity

All known octapeptides contain a bulky hydrophobic residue (phenylalanine, leucine or isoleucine) at position − 8, and a small hydroxylated residue (serine or threonine) or glycine at position − 5, relative to the MIP cleavage site [19,20] (Fig. 2). Small hydroxylated residues are frequently present at positions − 7 and − 6, R-X↓(F/L/I)-(S/X)-(S/T/X)-(T/S/G)-X-X-X-X↓ [21]. Octapeptide-containing precursors cannot be processed to the mature form by mitochondrial fractions containing MIP activity but devoid of MPP activity because the octapeptide must be at the substrate N-terminus in order to be accessible to MIP [36,44]. The presence of a bulky hydrophobic residue at − 8 is critical for substrate recognition by MIP, while there are less stringent requirements at the other positions (− 7 to − 1), suggesting that some hydrophobic interaction may occur between the octapeptide N-terminus and the MIP substrate binding site and that the rest of the octapeptide is required to stabilize this interaction [119,124].

Extensive surveys of octapeptide-containing precursors have not revealed any obvious consensus on the C-terminal side of MIP cleavage sites [19–21], although it has been suggested that some structural determinant in the mature portion may play a role in the substrate specificity [44,126,127].

4.4. Biochemical properties

MIPs from different species are soluble monomers of 70–75 kDa [120–122,128]. The enzyme is not abundant (~ 0.04% of total mitochondrial proteins in the case of RMIP) and is unstable, characteristics that resulted in relatively low yields when RMIP was first purified from mitochondrial matrix [128]. Overexpression in *S. cerevisiae* and affinity purification have been later used to produce larger quantities of YMIP [129]. Purified RMIP showed a pH optimum between 6.6 and 8.9, was strongly inhibited by 0.01 mM EDTA and fully reactivated by addition of 0.1–1 mM Mn^{2+}. Other metal ions either stimulated (Mg^{2+} or Ca^{2+}) or fully inhibited (Co^{2+}, Fe^{2+}, or Zn^{2+}) the enzyme at a metal concentration of 1 mM. This inhibition could be reversed by higher concentrations of Mn^{2+}. Interestingly, RMIP was inactivated by *N*-ethylmaleimide (NEM) and other thiol-group reagents [128]. Both metal and thiol dependence have also been reported for YMIP [129].

MIP sequences are homologous to the thimet oligopeptidase (TOP) family of metalloproteases that are also characterized by thiol dependence [130]. MIPs and TOPs contain a highly conserved domain, F-H-E-x-G-H-$(x)_2$-H-$(x)_{12}$-G-$(x)_5$-D-$(x)_2$-E-x-P-S-$(x)_3$-E, including a putative zinc-binding site, H-E-x-x-H, as well as one histidine and two glutamic acid residues [79,121] (Fig. 7). In addition, these proteins are enriched in cysteine residues. For example, RMIP, HMIP, YMIP, and SMIP contain 18, 16, 17 and 8 cysteine residues, respectively. Two of these residues are highly conserved, and one of them resides within the metal-binding domain [129] (Fig. 7). When single amino acids within the H-E-x-x-H motif, or a glutamic acid 24 residues C-terminal from the motif (Fig. 7) were replaced by other amino acids, there was loss of YMIP function in vivo, consistent with the metal dependence exhibited by RMIP and YMIP in vitro. In contrast, single or double replacements of the two conserved cysteines with serine or valine residues had no effect on YMIP activity in vivo, indicating that the two cysteines are not required for global peptidase function. Purified YMIP variants carrying such replacements, however, were found to be less stable and more sensitive to thiol-blocking agents than the wild-type protein [129]. These findings suggest that cysteine residues may affect MIP function in at least two ways: by forming one or more S–S bonds, which may be important to MIP stability, or by forming one or more Mn^{2+} binding sites, which may play a role in MIP activity. The latter possibility is suggested by the fact that recombinant YMIP is activated by Mn^{2+}, and that this effect is enhanced by DTT, an SH-reducing agent (G.I., unpublished results).

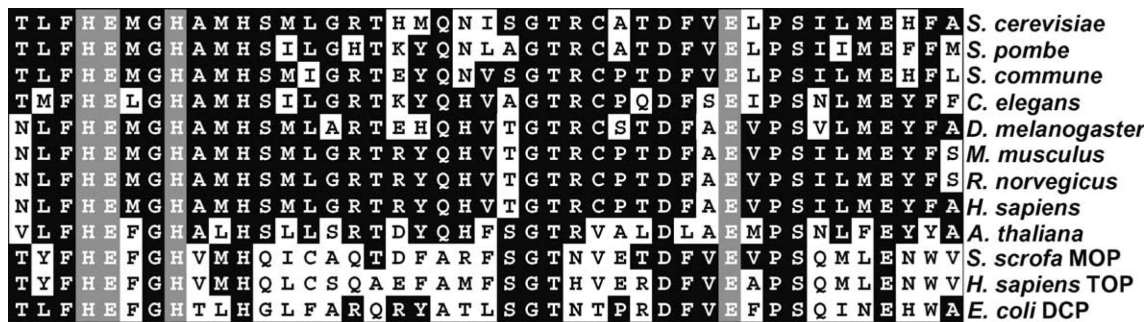

Fig. 7. Putative active site of MIP. Amino acids believed to be involved in metal binding are highlighted in gray. *S. cerevisiae* (P35999); *S. pombe* (T38081); *S. commune* (AAA93531); *C. elegans* (CAC44308); *D. melanogaster* (AAF57312); *M. musculus* (BAB30770); *R. norvegicus* (NP112314); *H. sapiens* (AAC51231); *A. thaliana* (NP199967); *Sus scrofa* mitochondrial oligopeptidase (Q02038); *H. sapiens* thimet oligopeptidase (AAA82607); *E. coli* peptidyl-dipeptidase (P24171).

4.5. Identification and sequence analysis of MIP homologues

The 2337 bp RMIP cDNA was first isolated and found to encode a protein of 710 amino acids with a presequence of 33 amino acids [119]. A typical R-2 motif was identified at the mature N-terminus and in vitro processing showed that the MIP precursor is cleaved to the mature form by MPP. In *S. cerevisiae*, the *MIP1* gene (redesignated *OCT1*) specifies a mitochondrial precursor of 772 amino acids with a predicted presequence of 37 amino acids also with a typical MPP cleavage site [120]. Additional cDNAs were isolated from the basidiomycete fungus *Schizophyllum commune* [121] and human liver [122]. The *S. cerevisiae OCT1* gene has no introns while the *S. commune* SMIP gene consists of four exons of 523, 486, 660 and 629 bp, separated by three small introns of 79, 50 and 53 bp, respectively [121]. The human *MIPEP* gene spans 57 kbp and consists of 19 exons encoding a mitochondrial precursor protein of 713 amino acids [122]. A 35-amino-acid-long presequence is encoded by exon 1, and the entire metal-binding domain by exon 13. The exon–intron structure of *MIPEP* is quite different from those of mitochondrial oligopeptidase (EC 3.4.24.16) and TOP (EC 3.4.24.15) [131], in spite of the sequence homology among these proteins and their almost identical catalytic domains [121,130]. The *MIPEP* transcript is expressed differentially and predominantly in tissues with high oxygen consumption (heart, skeletal muscle and several different regions of the brain) [122,132]. A polymorphic $(CA)_n$ repeat was identified in intron 4 and used to genetically map *MIPEP* within a 7 cM interval between markers D13S283 and D13S217 on chromosome 13q12 [132].

In addition to the MIPs listed above, a number of putative homologues can be found in the sequence databases, some of which are shown in Figs. 7 and 8. There is approximately 35% overall identity and 55% similarity among these sequences. Homology is particularly high around the putative active site toward the protein's C-terminus (Fig. 7). As mentioned above, MIP sequences show 20–24% identity and 40–47% similarity to the TOPs and together constitute the M3 family of metallopeptidases [79]. TOPs cleave short peptide substrates, and whereas mitochondrial oligopeptidase resides in the mitochondrial intermembrane space, most TOPs are localized to the cytoplasm [130].

4.6. Biologic function and possible role in mitochondrial disease

In *S. cerevisiae*, octapeptide-containing proteins include (i) subunits of pyridine- and flavin-linked dehydrogenases; (ii) iron–sulfur cluster-containing proteins and other nucleus-encoded subunits of respiratory chain complexes;

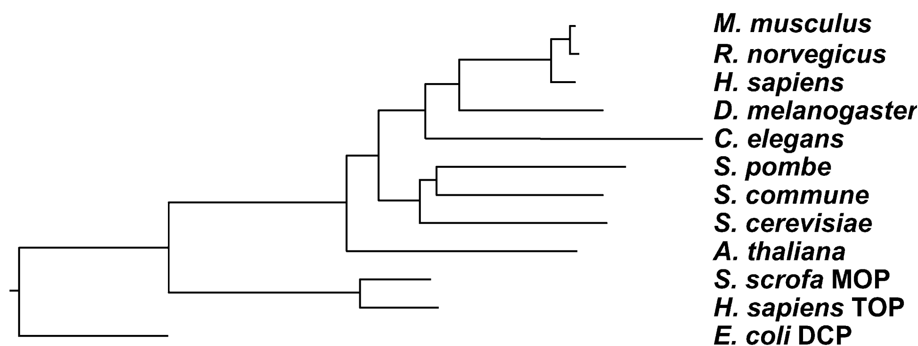

Fig. 8. Phylogenetic tree of MIP sequences. The tree was generated as described in the legend of Fig. 3. Sequence accession numbers are given in the legend of Fig. 7.

(iii) proteins required for replication and expression of mitochondrial DNA (mtDNA); and (iv) ferrochelatase, the enzyme that catalyzes iron attachment in the last step of heme synthesis [21]. The roles played by these proteins suggest that YMIP is important for oxidative metabolism, and accordingly, an *oct1*Δ mutant was found to exhibit a respiratory-deficient phenotype with multiple electron transport chain enzyme deficits and loss of mtDNA [120]. RMIP, SMIP or HMIP could all rescue this phenotype, indicating that the role played by MIP in oxidative metabolism is conserved in eukaryotes [121,132]. The importance of this role is underscored by the observation that disruption of the mouse MIP gene results in early embryonic lethality around 8.5 d.p.c (G.I., unpublished results).

An additional study showed that YMIP stimulates mitochondrial iron uptake, probably due to the fact that YMIP catalyzes the maturation of ferrochelatase and iron–sulfur cluster-containing proteins [21,123]. Interestingly, the presence of YMIP was found to exacerbate the mitochondrial iron accumulation that results from genetic inactivation of yeast frataxin [123], a conserved mitochondrial protein required for cellular iron homeostasis, a deficiency of which causes the human disease, Friedreich ataxia (reviewed in Ref. [86]). This finding has opened the possibility that *MIPEP* could be one of the loci that influence the clinical manifestations of Friedreich's ataxia or even be responsible for phenocopies of the disease [133].

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