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Matrix Processing Peptidase of Mitochondria

STRUCTURE-FUNCTION RELATIONSHIPS*

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The mitochondrial processing peptidase (MPP) and the processing enhancing protein (PEP) cooperate in the proteolytic cleavage of matrix targeting sequences from nuclear-encoded mitochondrial precursor proteins. We have determined the cDNA sequence of *Neurospora* MPP after expression cloning. MPP appears to contain two domains of approximately equal size which are separated by a loop-like sequence. Considerable structural similarity exists to the recently sequenced yeast MPP as well as to *Neurospora* and yeast PEP. Four cysteine residues are conserved in *Neurospora* and yeast MPP. Inactivation of MPP can be achieved by using sulfhydryl reagents. MPP (but not PEP) depends on the presence of divalent metal ions for activity. Both MPP and PEP are synthesized as precursors containing matrix targeting signals which are processed during import into mitochondria by the mature forms of MPP and PEP.

The amino-terminal presequences of nuclear-encoded precursor proteins are necessary for targeting these precursors to mitochondria (for reviews see Attardi and Schatz, 1988; Hartl *et al.*, 1989). These target sequences are removed during or after import by a processing enzyme located in the mitochondrial matrix (Böhni *et al.*, 1980; Hurt *et al.*, 1984; Horwich *et al.*, 1985, 1986; Emr *et al.*, 1986; Keng *et al.*, 1986; Kalousek *et al.*, 1988).

A comparison of the various presequences determined so far reveals very few common structural features (von Heijne, 1986; Roise *et al.*, 1986; Vassarotti *et al.*, 1987). All presequences have a relatively high content of positive charges and may have a tendency to form amphipathic α-helical structures when inserted into a membrane. In many presequences but not in all there is an abundance of hydroxylated amino acids (serine and threonine). Otherwise, they are rather diverse, both with regard to the sequences of the targeting peptides and to the sequences around the cleavage sites. A common theme of the cleavage sites, however, seems to be the presence of an arginine residue 2 residues upstream of the peptide bond to be hydrolyzed (Nicholson and Neupert, 1988; von Heijne, 1988; Hartl *et al.*, 1989).

The catalytic specificity of the matrix processing enzyme thus appears to be of considerable interest. On the one hand, the peptidase acts on hundreds or thousands of rather diverse presequences and cleavage sites; on the other hand, it makes a single and specific cut (Ou *et al.*, 1989).

The activity of a matrix processing enzyme has been determined in mitochondria from different organisms and has been shown to be metal-dependent (Böhni *et al.*, 1980; McAda and Douglas, 1982; Miura *et al.*, 1982; Conboy *et al.*, 1982; Schmidt *et al.*, 1984). The enzyme was first purified from *Neurospora crassa* (Hawlitschek *et al.*, 1988). Two proteins are required for proteolytic activity, the mitochondrial processing peptidase (MPP)[1] which appears to be the catalytic component, and the processing enhancing protein (PEP). *Neurospora* MPP and PEP have apparent molecular masses of 57 kDa and 52 kDa, respectively. The enzyme was subsequently isolated from yeast (Yang *et al.*, 1988). It turned out that MPP is the product of the gene MIF2 or MAS2 (Pollock *et al.*, 1988; Jensen and Yaffe, 1988) and PEP the product of the gene MAS1 or MIF1 (Witte *et al.*, 1988).[2] MPP and PEP were found to be structurally related, with a sequence identity of 20% (Pollock *et al.*, 1988; Yang *et al.*, 1988). Moreover, core proteins 1 and 2 (also called subunit I and II), the products of the genes COR1 (Tzagoloff *et al.*, 1986) and COR2 (Oudshoorn *et al.*, 1987) of ubiquinol cytochrome *c* reductase, are members of the protein family which includes both MPP and PEP. In *Neurospora,* PEP and core1 turned out to be structurally identical to each other (Schulte *et al.*, 1989).

In an attempt to obtain further insight into the role of MPP, we have cloned the cDNA from *N. crassa* and have compared it with the yeast MPP sequence. Several highly conserved regions are found which may have a particular role in the catalytic activity in MPP and which are not present in other members of the MPP/PEP/core family. Most interestingly, 4 cysteine residues were found in identical positions in the two MPPs. Experiments with sulfhydryl reagents show that reactive cysteines indeed have an important function in MPP but not in PEP. Moreover, MPP is the Mn^{2+} ion-binding component responsible for the metal ion requirement of the processing activity. Finally, the data suggest that MPP is comprised of two domains of roughly equal size which are divided by a loop-like structure with an unusual amino acid composition.

EXPERIMENTAL PROCEDURES

Synthesis of a Sized cDNA Library from N. crassa—cDNA samples were prepared in reactions containing 5 μg of isolated poly(A)$^+$ mRNA

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05484.

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[1] The abbreviations used are: MPP, mitochondrial processing peptidase; PEP, processing enhancing protein; bp, base pair; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; nt, nucleotide; NEM, *N*-ethylmaleimide; MOPS, 4-morpholinepropanesulfonic acid.

[2] A. Horwich, unpublished results.

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of *N. crassa* (Kleene *et al.*, 1987) with the cDNA synthesis kit including *Eco*RI-adaptors of Pharmacia LKB Biotechnology Inc. following the standard protocol. Three samples were combined, and cDNA was fractionated according to length by electrophoresis in an 0.8% agarose gel. The gel was divided and five fractions of cDNA corresponding to (*a*) 300–900 bp, (*b*) 900–1500 bp, (*c*) 1500–2000 bp, (*d*) 2000–3000 bp, and (*e*) >3000 bp were obtained following electro-elution of the cDNAs. The isolated fragments were ligated with 2 μg (*a* and *b*) or 1 μg (*c*–*e*) of λgt11-*Eco*RI arms (Pharmacia LKB Biotechnology Inc.), respectively. Immediately after packaging (Gigapack Gold, Stratagene) the libraries were amplified in *Escherichia coli* strain Y1088. The number of different phages before amplification were (*a*) $1.5 \cdot 10^6$, (*b*) $5 \cdot 10^5$, (*c*) $9 \cdot 10^4$, (*d*) $2.8 \cdot 10^5$, (*e*) $8 \cdot 10^4$. The libraries had a titer of 1–5 pfu/pl and were stored in SM (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris/Cl pH 7.5, 1% gelatin) containing 1% $CHCl_3$ and 0.02% NaN_3 at 4 °C or at −70 °C with additional 7% Me_2SO.

Screening Procedure and Sequencing Strategy—For immunoscreening $5 \cdot 10^4$ pfu in Y1090 were grown in top agarose (1% Bactoagar, 0.625% yeast-extract, 0.1 M NaCl, 10 mM $MgSO_4$, 0.7% agarose) on agar plates with a diameter of 140 mm. In total $2.5 \cdot 10^5$ pfu of library C were analyzed. Screening was performed as described previously by Young and Davis (1983). MPP antibody was diluted 1:1000. For detection of positive clones, we used anti-rabbit IgG antibody coupled to α-peroxidase (Sigma; Tsung *et al.*, 1983). Positive clones were picked and rescreened at a density of 200 plaques/94-mm plate. In a second rescreen we tested them for homogeneity. λ-DNA was isolated in small scale preparations and cleaved with *Eco*RI. The cDNA inserts were subcloned into the *Eco*RI site of pGEM3.

Screening for full-length clones was done by plaque hybridization using the ^{32}P-labeled insert of the antibody-positive clone pMw2 as a probe. $5 \cdot 10^4$ pfu were grown on five agar plates (94 mm) in Y1088 at 37 °C overnight. The plaque DNA was fixed *in situ* on Nylon membranes (Amersham Corp.; Benton and Davis, 1977). Hybridization and washing was carried out following the standard protocol. We detected several positive clones, and two of them (pMk1 and pMk2) were finally purified and subcloned into pGEM3. A genomic library cloned in pBR322 was screened by colony hybridization using the same probe.

Supercoil sequencing (Chen and Seeburg, 1985) was performed by the dideoxy-chain termination method (Sanger *et al.*, 1977). Shortened clones were prepared by exonuclease III/nuclease S1 treatment (Henikoff, 1984), by digestion with restriction enzymes (Boehringer Mannheim) that cut both the polylinker and the cDNA (*Hin*dII, *Hin*dIII, *Sal*I, *Sma*I) followed by religation, or by subcloning of fragments of the cDNA into pUC19. Parts of the cDNA and genomic clones were sequenced by using MPP-specific synthetic oligonucleotide primers.

In Vitro Transcription, Translation, and Processing—Full-length MPP cDNA (pMk2) as well as other precursor protein cDNAs were cloned in pGEM3. ppreMPP160 was constructed by cleaving pMk2 with *Eco*RI and *Hin*dIII, blunt-end formation with Klenow enzyme, and cloning the fragment of the 5′-end into a pGEM3-vector, which had been digested with *Hin*dIII and *Xba*I and blunt-ended with Klenow enzyme. By this way the TAG codon in the *Xba*I-site was placed into the MPP reading frame. The plasmids were transcribed with Sp6-RNA polymerase (Promega). Precursor proteins were synthesized in rabbit reticulocyte lysate containing radiolabeled amino acids (Pelham and Jackson, 1976).

The processing peptidase assay was carried in the presence of 1% Triton X-100, 30 mM Tris/Cl, pH 8.2, 1 mM $MnCl_2$ and 1 mM phenylmethanesulfonyl fluoride (PMSF) in a final volume of 15 μl. Partially purified MPP (25 ng) and purified PEP (25 ng) were used. The reaction was started with 1.5 μl of lysate containing the precursor of the β subunit of F_1-ATPase ($pF_1\beta$) synthesized from the cDNA in plasmid pGEM as substrate. After 30 min at 25 °C, the processing reaction was stopped by addition of 15 μl of Laemmli buffer (2-fold concentrated; Laemmli, 1970). Processing products were analyzed by SDS-polyacrylamide gel electrophoresis, fluorography, and laser densitometry.

In Vitro Import into Mitochondria—Mitochondria were prepared from freshly harvested *N. crassa* (Hennig and Neupert, 1983) by differential centrifugation in 250 mM sucrose, 2 mM EDTA, 10 mM MOPS/KOH, pH 7.2 ("SEM") and 1 mM PMSF (Pfanner and Neupert, 1985). Mitochondria (20 μg of protein) were incubated in 3% bovine serum albumin, 2.5 mM $MgCl_2$, 80 mM KCl, 10 mM MOPS/KOH pH 7.2, 250 mM sucrose, 2 mM NADH with 10 μl of lysate containing *in vitro* synthesized precursor protein for 30 min at 25 °C. After reisolation, mitochondria were incubated in 100 μl SEM containing 4 μg of proteinase K for 25 min at 0 °C (Pfanner and Neupert, 1986). Proteolysis was stopped by adding 2 μl of 0.1 M PMSF. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Radiosequencing—preMPP160 was synthesized in reticulocyte lysate containing [^{35}S]methionine, [^3H]glutamic acid, or [^3H]valine. Mature sized MPP160 (mMPP160) was prepared (i) by import into mitochondria (3.6 mg of protein) in a volume of 9.6 ml and subsequent proteinase K treatment and lysis of reisolated mitochondria in 400 μl of lysis buffer (30 mM sodium phosphate, pH 8.2, 2% SDS) for 5 min at 56 °C, or (ii) by pelleting the precursor with 67% ammonium sulfate (to separate from hemoglobin), processing with purified MPP/PEP in a volume of 100 μl, gel electrophoresis and elution of the band corresponding to mMPP160 three times in 300 μl of lysis buffer at 22 °C for 1 h. Solutions containing 25,000 cpm ([^{35}S]methionine), 30,000 cpm ([^3H]glutamic acid), 18,000 cpm ([^3H]valine, imported), and 60,000 cpm ([H]valine, *in vitro* processed) were analyzed by solid-phase Edman degradation as described previously (Wachter *et al.*, 1973).

Purification of Processing Peptidase—Purification of PEP was carried out as described by Schulte *et al.* (1989). For purification of MPP a total protein extract of *N. crassa* hyphae (100 g) was prepared according to Hawlitschek *et al.* (1988). The initial chromatographic steps, namely on DEAE-cellulose (Whatman), metal chelate affinity Sepharose 6B (Pharmacia LKB Biotechnology Inc.), and PEI-cellulose (Sigma) were performed as described before (Hawlitschek *et al.*, 1988). The resulting fractions from the PEI-cellulose chromatography containing MPP were then applied to a hydroxyapatite column (Bio-Rad; 2.5 × 10 cm) and chromatographed with a linear 0–200 mM sodium phosphate gradient. MPP eluted at a phosphate concentration of 160–180 mM. The pooled fractions were applied to a Mono Q column (Pharmacia HR 5/5). Proteins were eluted with a linear salt gradient from 0 to 300 mM and MPP eluted at a salt concentration of 110 mM NaCl. The yield of MPP was 1% (≈5 μg of protein). This preparation was free of PEP as judged by Western blotting. All steps were carried out at 4 °C and monitored by SDS-gel electrophoresis and Western blotting (Burnette, 1981).

Immunodecoration was carried out with antibody against MPP and visualized using anti rabbit IgG antibody coupled to alkaline phosphatase (Blake *et al.*, 1984).

RESULTS

cDNA Cloning, Sequencing, and Predicted Amino Acid Sequence of Neurospora MPP—A cDNA library of *N. crassa* containing cDNA inserts in the range of 1500–2000 bp cloned in λgt11 (Young and Davis, 1983) was screened by using an antibody probe against *Neurospora* MPP. We examined $2.5 \cdot 10^5$ phage plaques and obtained five positive clones. The clones were isolated and the cDNAs were subcloned into the plasmid vector pGEM3. Sequence analysis of the 5′- and 3′-ends showed that all clones were identical and were termed pMw2. The cDNA-insert of pMw2 was 1902 bp in length. The library was then rescreened with the ^{32}P-labeled insert of pMw2. Two further clones (pMk1, pMk2) were analyzed and subcloned into pGEM3. pMk1 started 198 bp downstream of the 5′-end of pMw2 and included a fragment of a poly(A) tail containing 3 adenine residues. pMk2, compared with pMw2, contained an additional 126 residues at the 5′-end, including the proposed start codon, and lacked only a few residues at the 3′-end. pMk2 most likely represented a full-length clone. A genomic DNA-library in pBR322 was also screened and genomic clones were isolated.

The complete pMw2 insert was sequenced while the other clones were sequenced only in part. An outline of the sequencing strategy is given in Fig. 1. The cDNA consists of 2037 bp (Fig. 2). Translation most likely starts at the ATG codon at nt 41 and stops at nt 1772, so that the open reading frame codes for a polypeptide of 577 amino acids with a predicted molecular weight of 62,940. The translation initiation site fits well with the consensus sequence for eukaryotes (GCAGCCATGC *versus* GCCACCATGG (Kozak, 1984)). The

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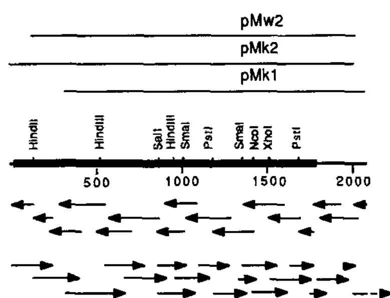

FIG. 1. **Strategy for sequencing of *Neurospora* MPP-cDNA.** Restriction sites used for subcloning and deletions are indicated. The *upper lines* indicate the extensions of the analyzed cDNA clones. The *arrows* represent the directions and lengths of the sequences determined. Subclones of the cDNA in both orientations were prepared in pGEM3, and they were sequenced by the dideoxy method. Shortened clones for overlapping sequence information were obtained by exonuclease III/S1 nuclease treatment, by subcloning of cDNA fragments into pUC19, by deletion of parts of the cDNA using the indicated restriction sites, and by use of MPP-specific oligonucleotide primers. The open reading frame is indicated by the *filled bar* in the central line.

3′-untranslated region consists of 265 residues and includes a putative polyadenylation signal (AATATA) 24 bp upstream of the poly(A) tail. The exact start of the tail was determined by sequencing of a genomic clone.

Comparison with Related Protein Sequences—The amino acid sequence of *Neurospora* MPP was compared with the sequences of the protein family including, so far, yeast MPP, PEP, and core proteins 1 and 2 of the cytochrome *c* reductase, and *Neurospora* PEP (Schulte *et al.*, 1989). Compared with yeast MPP there is a sequence identity of 43.5%. An alignment of the two MPP sequences is presented in Fig. 3. We found homologies in all parts of the protein with the exception of a serine-rich region in the center of the *Neurospora* sequence, which has no counterpart in the yeast protein. Without taking into account this region the identity is 48.3%. To verify this unusual sequence, three independent cDNA clones and one genomic clone were isolated, and the sequence in this region was confirmed. Therefore, it is unlikely that there was erroneous cDNA synthesis. In fact, the protein obtained by *in vitro* transcription, translation, and processing had the same mobility upon SDS-polyacrylamide gel electrophoresis as the purified MPP (not shown). Computer analysis predicted a high level of flexibility for this stretch, and both *Neurospora* and yeast MPP show a high frequency of proline residues in this area. This sequence may have originated from an intron which has lost one of its splice sites during evolution. Possible 5′-ends of such a putative intron are at nt 793 (GTACTT) and at nt 828 (GTCTCT). The consensus sequence for *Neurospora* is GTAXGT (Bowman *et al.*, 1988). The 3′-end would be at nt 1014 (TAG) (consensus, PyAG) with CTCAC (consensus, CTPuAC) corresponding to the branch site 15 bp upstream (Fig. 2).

Several striking similarities between the *Neurospora* and yeast enzyme exist: first, there is a stretch of 33 identical residues starting at amino acid position 372. Most remarkable are 4 glycine residues which are surrounded by uncharged amino acids. This rather hydrophobic area has a predicted high flexibility in the otherwise hydrophilic protein and may therefore be located in the interior of the molecule. It may be relevant that this motif is not present in PEP and in core protein 1 of cytochrome *c* reductase. This region therefore may be important for the activity of the protein. Second, a highly conserved region extends from amino acid 467 to 506. This region is hydrophilic and is a common element of the

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   1                             cccacattacgctgccgcatcacaattccttgttgcagcc

  41 ATGCTGAATCGCTTCCGGCCAGCGCGGCTAGTAGCCCAATCCTCCAGATGCTTGCCCTTG
      M  L  N  R  F  R  P  A  R  L  V  A  Q  S  S  R  C  L  P  L  20

 101 ACGAGGGCGCGGGCAGGTCCCTTGCCCGTTAACAATGCCAGGACTTTGGCTACGAGAGCC
      T  R  A  R  A  G  P  L  P  V  N  N  A  R  T ▲L  A  T  R  A  40

 161 GCTGCTGTCAACACCAAGGAACCGACCGAACGCGACAACATCACCACTCTCTCCAATGGT
      A  A  V  N  T  K  E  P  T  E  R  D  N  I  T  T  L  S  N  G  60

 221 GTCCGTGTCGCTTCCGAGGACCTTCCCGATGCCTTCTCCGGTGTAGGTGTCTACATCGAC
      V  R  V  A  S  E  D  L  P  D  A  F  S  G  V  G  V  Y  I  D  80

 281 GCGGGGTCCCGATATGAGAACGACTATGTCCGGGGTGCCAGTCACATCATGGACCGGCTA
      A  G  S  R  Y  E  N  D  Y  V  R  G  A  S  H  I  M  D  R  L 100

 341 GCCTTCAAGTCTACAAGTTCGAGGACTGCGGACGAAATGCTCGAAACTGTTGAGAAGCTC
      A  F  K  S  T  S  S  R  T  A  D  E  M  L  E  T  V  E  K  L 120

 401 GGTGGTAACATTCAGTGCGCTTCTTCGCGCGAGTCTATGATGTACCAGGCGGCCACCTTC
      G  G  N  I  Q  C  A  S  S  R  E  S  M  M  Y  Q  A  A  T  F 140

 461 AACAAGGCTATTCCCACCGCTGTTGAGCTCATGGCCGAGACCATCCGCGATCCCAAGCTT
      N  K  A  I  P  T  A  V  E  L  M  A  E  T  I  R  D  P  K  L 160

 521 ACGGACGAGGAGCTGGAGGGACAGATCATGACGGCGCAATATGAGGTCAACGAGATCTGG
      T  D  E  E  L  E  G  Q  I  M  T  A  Q  Y  E  V  N  E  I  W 180

 581 TCCAAGGCCGAACTGATCCTGCCCGAGTTGGTGCACATGGCTGCCTTCAAGGACAACACT
      S  K  A  E  L  I  L  P  E  L  V  H  M  A  A  F  K  D  N  T 200

 641 CTTGGCAACCCGTTGCTTTGTCCCAAGGAGAGGTTGGATTACATCAACCGGGATGTCATC
      L  G  N  P  L  L  C  P  K  E  R  L  D  Y  I  N  R  D  V  I 220

 701 CAAACATACCGCGACGCTTTCTACAGGCCCGAGCGCCTTGTTGTTGCCTTTGCTGGTGTG
      Q  T  Y  R  D  A  F  Y  R  P  E  R  L  V  V  A  F  A  G  V 240

 761 CCTCATGAGAGGGCCGTCAAGCTCGCAGAGAAGTACTTTGGTGATATGAAGGCCTCCGAT
      P  H  E  R  A  V  K  L  A  E  K  Y  F  G  D  M  K  A  S  D 260

 821 GCTCCCGGTCTCTCGAGGACAGGTTCCGAAACCTCCGTCGACTCGCTAGTGTCCGAGTCC
      A  P  G  L  S  R  T  G  S  E  T  S  V  D  S  L  V  S  E  S 280

 881 AGCGAGGCCTCGAGTGAATCTTCATCATCCTCCTCGGACTCTTCCGAGTCGAGTGGCGGG
      S  E  A  S  S  E  S  S  S  S  S  S  D  S  S  E  S  S  G  G 300

 941 CTGCTCTCCAAGCTTTTCTCTCCCAAGGCCAAGAAAGCCACCCCCAACCCCTTCCTCACC
      L  L  S  K  L  F  S  P  K  A  K  K  A  T  P  N  P  F  L  T 320

1001 CGGGTACCTATTAGCACCGAAGACTTGACTCGGCCTGCTCACTACACAGGCGGTTTCCTC
      R  V  P  I  S  T  E  D  L  T  R  P  A  H  Y  T  G  G  F  L 340

1061 ACCCTCCCATCACAGCCCCCACCGCTCAACCCCAACCTTCCCACATTTACTCACATACAG
      T  L  P  S  Q  P  P  P  L  N  P  N  L  P  T  F  T  H  I  Q 360

1121 CTCGCCTTCGAGGGCCTCGCCATCTCGGACGACGACATCTACGCCCTCGCCACCCTGCAG
      L  A  F  E  G  L  A  I  S  D  D  D  I  Y  A  L  A  T  L  Q 380

1181 ACCCTCCTCGGCGGCGGCGGCTCCTTCTCTGCCGGCGGTCCCGGCAAGGGCATGTACTCG
      T  L  L  G  G  G  G  S  F  S  A  G  G  P  G  K  G  M  Y  S 400

1241 CGTCTCTACACTAACGTTCTCAACCAGCACGGCTGGGTTGAGTCCTGCGTGGCCTTCAAC
      R  L  Y  T  N  V  L  N  Q  H  G  W  V  E  S  C  V  A  F  N 420

1301 CACTCATACACGGACTCGGGTCTCTTCGGCATCGCCGCCTCGTGCTACCCGGGTCGCACC
      H  S  Y  T  D  S  G  L  F  G  I  A  A  S  C  Y  P  G  R  T 440

1361 CTGCCCATGCTCCAGGTCATGTGCCGCGAGCTGCACGCCCTCACCACCGACCATGGCTAC
      L  P  M  L  Q  V  M  C  R  E  L  H  A  L  T  T  D  H  G  Y 460

1421 TCGGCCCTGGGCGAGCTCGAGGTTTCGCGCGCCAAGAACCAGCTCCGCAGCAGCCTCCTG
      S  A  L  G  E  L  E  V  S  R  A  K  N  Q  L  R  S  S  L  L 480

1481 ATGAACCTCGAGAGCCGCATGGTCGAGCTCGAGGATCTGGGCCGCCAAGTTCAGGTTCAC
      M  N  L  E  S  R  M  V  E  L  E  D  L  G  R  Q  V  Q  V  H 500

1541 GGTCGCAAGATCCCGGTCCGCGAGATGACGCGCCGTATCAACGAGCTGACGGTCAAGGAC
      G  R  K  I  P  V  R  E  M  T  R  R  I  N  E  L  T  V  K  D 520

1601 CTCCGAAGGGTCGCTAAGCGCGTGGTTGGTGGCATGGCGAATAACGCCGGCCAGGGAAGC
      L  R  R  V  A  K  R  V  V  G  G  M  A  N  N  A  G  Q  G  S 540

1661 GGTGCGCCGACGGTGGTGCTGCAGGAGGCGACGGTGCAAGGACTCAAGACTACGGAGCTG
      G  A  P  T  V  V  L  Q  E  A  T  V  Q  G  L  K  T  T  E  L 560

1721 GGGTGGGATCAGATCCAGGATACAATTGCTCAGTGGAAGCTCGGTAGACGGTAAacgttt
      G  W  D  Q  I  Q  D  T  I  A  Q  W  K  L  G  R  R  *       577

1781 gtcaaggggaaaaaaagagtagggcgtggagaagttatgtaagaggagcgctgtattgaa

1841 cttggcgacacgcacacaccggaacgataaaggcgttttaggttccccacgagcataggg

1901 aagaggctagatggttgctctgtacaatcgcaactttttcttggtgagttatacaagatgt

1961 gtccaggtacatctttgccttaccatactgtacgatagcaatgaagattttctgaatata

2021 tcaaaagtcaaaagtca(a)N
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FIG. 2. **Nucleotide sequence (coding strand) of the *Neurospora* MPP cDNA and deduced amino acid sequence.** The *arrow* marks the cleavage site between presequence and mature protein. *Boxed* sequences indicate similarities to intron boundaries (*shadowed*) and to the branch site consensus (*light box*).

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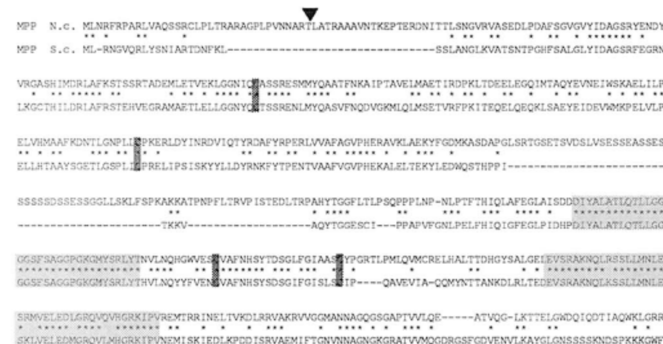

FIG. 3. **Sequence alignment of *Neurospora* and yeast MPP.** Identical residues are marked by *asterisk*. The 4 conserved cysteine residues and two strongly conserved regions are indicated by *shaded boxes*. The cleavage site of the matrix processing peptidase is marked by an *arrow*.

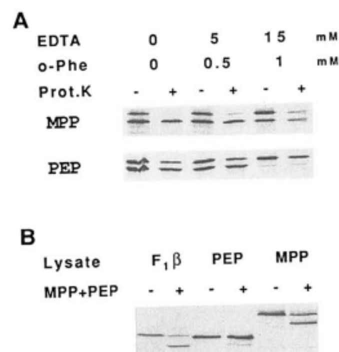

FIG. 4. *A*, Import of MPP and PEP into *Neurospora* mitochondria. Import was performed as described under "Experimental Procedures." The mitochondria, suspended in bovine serum albumin buffer including cold lysate and EDTA, 1,10-phenanthroline (*o-Phe*) were preincubated for 5 min at 25 °C. Then either ^{35}S-labeled precursor of MPP or PEP, synthesized in reticulocyte lysate, was added and incubated for 30 min at 25 °C. *B*, *In vitro* processing of precursors of MPP, PEP, and $F_1\beta$. Reticulocyte lysates containing *in vitro* synthesized precursors were incubated with approximately 50 ng of MPP and PEP in 30 mM Tris/Cl pH 8.2, 1% Triton X-100, 1 mM $MnCl_2$ and 1 mM PMSF for 30 min at 25 °C. Samples were analyzed by SDS-gel electrophoresis and fluorography.

MPP/PEP/core family. Third, there are 4 conserved cysteine residues (Cys-126, -207, -416, -435) in MPP of *Neurospora* and yeast.

We found no striking similarities to other proteins when we made an alignment against data banks. In particular, we compared the MPP sequences of *Neurospora* and yeast with several known proteases, especially cysteine and metalloproteases (Kamphuis *et al.,* 1985; Jongeneel *et al.,* 1989). The only similarity we found was to a well-conserved sequence in cysteine proteases. The motif His-Ala-Val-Thr-Ala-Ile-Gly-Tyr in stem bromelain (Golo *et al.,* 1980) is similar to the motif His-Ala-Leu-Thr-Thr-Asp-His-Gly-Tyr (amino acids 452–460) in *Neurospora* MPP. This motif, however, is not present in yeast MPP. Therefore it remains doubtful as to whether it is significant.

Both MPP and PEP Precursors Are Processed by Their Combined Mature Forms—The two components of the processing enzyme, MPP and PEP, are encoded in the nucleus. Recently, it has been reported that PEP is synthesized as a precursor and is processed to the mature form during import into mitochondria both in *Neurospora* and yeast (Hawlitschek *et al.,* 1988; Witte *et al.,* 1988). For yeast MPP it is so far unknown whether there is a cleavable signal sequence (Pollock *et al.,* 1988; Jensen and Yaffe, 1988). The amino terminus of *Neurospora* MPP contains a number of arginines as well as serine and threonine residues but no negative charges. These features are generally observed in mitochondrial targeting sequences.

We subcloned both MPP and PEP cDNA into pGEM3 vector and performed *in vitro* transcription with Sp6-RNA polymerase followed by *in vitro* translation in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The labeled MPP and PEP precursors obtained in this way could be imported into isolated *Neurospora* mitochondria. In both cases a protein with reduced molecular weight was formed and was found to be protected against protease added to the reisolated mitochondria (Fig. 4*A*). In the case of PEP, part of the unprocessed precursor was also protease-protected. In the presence of EDTA and 1,10-phenanthroline, which chelate metal ions and in this way inhibit the activity of the processing enzyme in the mitochondria (Schmidt *et al.,* 1984), proteolytic cleavage was blocked completely in the case of PEP and was reduced to about 50% in the case of MPP.

When the lysates containing labeled MPP and PEP precursors were incubated with purified MPP and PEP, defined molecular weight shifts were observed (Fig. 4*B*). Again the cleavage of the MPP precursor was more efficient than the cleavage of the PEP precursor. We conclude that MPP and PEP follow the general import pathway for nuclear-encoded mitochondrial proteins; they are synthesized as precursors in the cytosol and then processed during import into mitochondria by cooperation of their own mature forms.

The start of the mature MPP was determined by radiosequencing of the protein that had been (i) imported into and processed in mitochondria and (ii) processed by using purified processing peptidase. Since the full-length protein was labile during the sequencing procedure and no clear result was obtained, we used a truncated form of MPP (preMPP160). This contained the first 160 amino acids of the MPP precursor. preMPP160 was synthesized by *in vitro* transcription/translation in the presence of various radiolabeled amino acids.

Radiosequencing data on the processed form got from import into mitochondria are shown in Fig. 5*A*. After labeling with [^{35}S]methionine, we observed a peak at position 11, which corresponds to a lysine; since the protein was coupled to the solid support via the ϵ-amino groups of lysine residues, every lysine gives a peak. Labeling with [^{3}H]valine resulted in two peaks at position 8 (Val-8) and position 11 (Lys-11). When using [^{3}H]glutamic acid we observed peaks at position 11 and 15 (Glu-15). The putative signal for the predicted Glu-12 is likely hidden in the tail of the strong signal at position 11.

When preMPP160 radiolabeled with [^{3}H]valine and processed by the purified enzyme was analyzed, peaks at positions 8, 11, 26, and 28 were observed (Fig. 5*B*). They apparently correspond to Val-8, Lys-11, Val-26, and Val-28.

We conclude that the processing site is after amino acid position 35 of the precursor of MPP: Asn-Asn-Ala-Arg-Thr-∇-Leu-Ala-Thr-Arg. This cleavage site is in agreement with the consensus Arg-X-∇-Y (Nicholson and Neupert, 1988). It is, in fact, also very similar to the processing site in PEP which is Arg-Arg-Gly-∇-Leu-Ala-Thr (Hawlitschek *et al.,* 1988). It is further concluded that MPP/PEP are efficient in correctly processing MPP to its mature size.

The calculated molecular mass of mature MPP is then 59,058 daltons; this is somewhat higher than the apparent mass of 57 kDa determined by SDS-polyacrylamide gel electrophoresis of the purified enzyme (Hawlitschek *et al.,* 1988). Comparison of processed MPP from *in vitro* translation and purified MPP by SDS-polyacrylamide gel electrophoresis and

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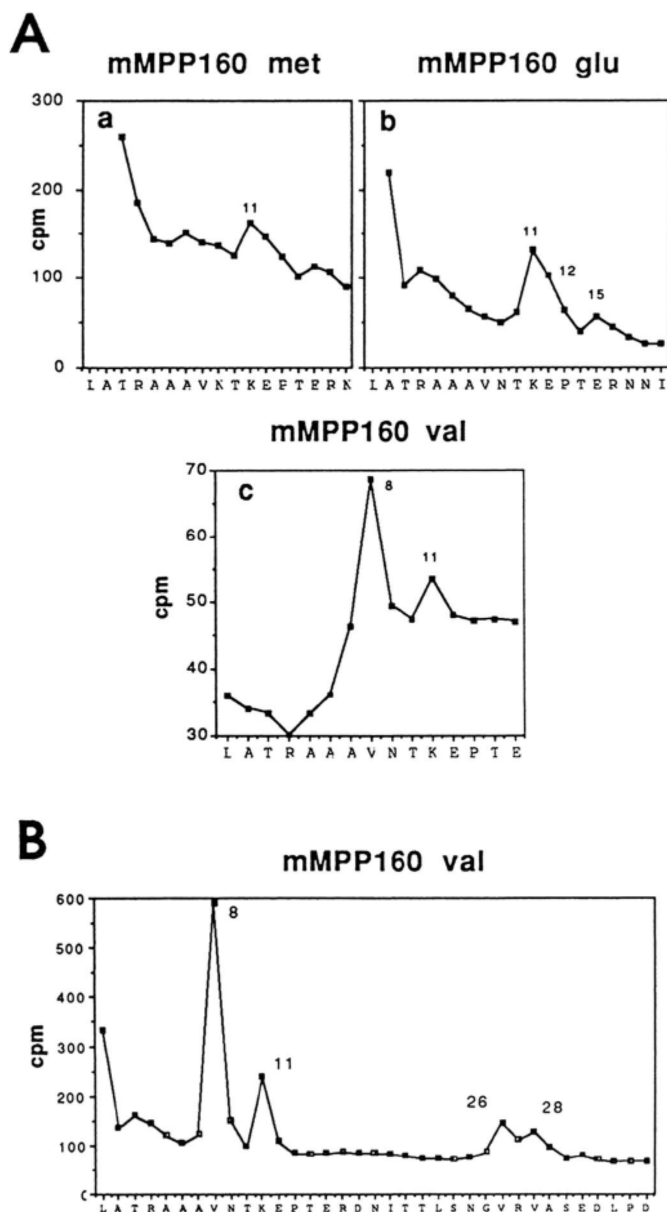

FIG. 5. **Radiosequencing of mature sized MPP160 (mMPP160).** Precursor of MPP160, a shortened form of MPP consisting of amino acids 1–160 of the precursor, was prepared in rabbit reticulocyte lysate containing radiolabeled amino acids. *A*, preMPP160 radiolabeled with [^{35}S]methionine (*a*), [^{3}H]glutamic acid (*b*), or [^{3}H]valine (*c*) in reticulocyte lysate was imported *in vitro* into mitochondria and subjected to radiosequencing as described under "Experimental Procedures." *B*, preMPP160 was synthesized *in vitro* in the presence of [^{3}H]valine. The precursor was precipitated with ammonium sulfate and processed with purified MPP and PEP as described under "Experimental Procedures." Proteins were separated by electrophoresis and visualized by fluorography. The band corresponding to mMPP160 was eluted and subjected to radiosequencing. The amino-terminal sequence of mature MPP160 deduced from the cDNA is displayed. Peaks corresponding to predicted amino acids are indicated. The first cycles in the runs of [^{35}S]methionine and [^{3}H] glutamic acid revealed high levels of free amino acids and are not shown. The heights of the peaks occurring at positions where lysines are found reflect the incidence at which a certain lysine was immobilized on the solid support; Lys-11 is near the cleavage site of the processing enzyme and may therefore be exposed.

Western blot analysis revealed identical apparent molecular masses (not shown).

Inactivation of MPP by Thiol Reagents—To address the question of whether cysteines are involved in processing of mitochondrial precursor proteins, a mitochondrial extract was treated with the sulfhydryl reagent *N*-ethylmaleimide (NEM). As shown in Fig. 6, the catalytic activity was largely abolished (*lane 2*). When NEM had been preincubated with dithioerythritol before the mitochondrial extract was added, the processing activity was fully preserved (*lane 3*). To determine if NEM specifically affects only one of the two components which are responsible for processing activity, purified PEP and MPP were separately treated with 10 mM NEM. The processing activity was strongly reduced if MPP was treated with NEM whereas the pretreatment of PEP with NEM had no effect on the catalytic activity (*lanes 5* and *8*). The hydrophilic sulfhydryl reagents iodoacetic acid and iodoacetamide also inhibited the catalytic activity of MPP, but the extent of inhibition was much less than that observed by the hydrophobic reagent NEM. 10 mM iodoacetate and iodoacetamide inhibited MPP by 67 and 35%, respectively, as compared with 95% inhibition with 10 mM NEM. MPP was completely inactivated by *p*-chloromercuric benzoate at a concentration of 0.01 mM (not shown). The NEM sensitivity would indicate that the thiol groups of one or more of the cysteine residues in MPP are necessary for catalytic activity.

Specific reagents that inhibit enzymes of the class of cysteine proteases such as chicken cystatin (Barrett *et al.,* 1986) and epoxysuccinyl-leucyl agmatine (E 64) from *Aspergillus japonicus* (Rich, 1986), however, did not affect processing activity.

The Activity of MPP Is Metal-dependent—The processing activity in mitochondria and of the purified enzyme has been reported to be dependent on divalent metals such as Mn^{2+} (Böhni *et al.,* 1980; Hawlitschek *et al.,* 1988). Which of the two components of the processing peptidase, MPP or PEP, is responsible for this metal dependence? To investigate this we incubated each protein in the absence or presence of 1 mM $MnCl_2$. Then immunoprecipitation with antibodies against either MPP or PEP was carried out in the absence or presence of 1 mM $MnCl_2$. Processing activity was then determined by addition of desalted PEP to immunoprecipitated MPP and addition of desalted MPP to immunoprecipitated PEP.

Without further addition of Mn^{2+} to the assay system, processing was only observed if MPP had been pretreated with Mn^{2+}, but not if PEP had been pretreated with Mn^{2+} (Fig. 7, *lanes 2* and *4*). If Mn^{2+} was absent during pretreatment of MPP and PEP, processing activity was not observed (*lanes*

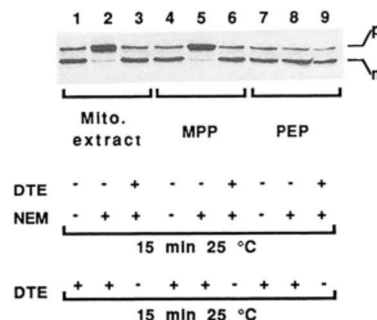

FIG. 6. **Inactivation of MPP by NEM.** Lysed mitochondria (0.5 μg of protein; *lanes 1–3*), MPP (25 ng; *lanes 4–6*) and PEP (25 ng; *lanes 7–9*) were treated with NEM as follows. Incubation was performed at 25 °C for 15 min with the following additions: (i) no NEM (*lanes 1, 4,* and *7*), (ii) 10 mM NEM (*lanes 2, 5,* and *8*), or (iii) a mixture of 20 mM dithioerythritol and 10 mM NEM (preincubated at 25 °C for 10 min; *lanes 3, 6,* and *9*). Dithioerythritol was then added in a second step at a final concentration of 20 mM to the controls and to the NEM-treated samples to inactivate unreacted NEM. This incubation was carried out at 25 °C for 10 min. Finally processing was tested with $F_1\beta$ as a substrate. *p*, precursor; *m*, mature $F_1\beta$.

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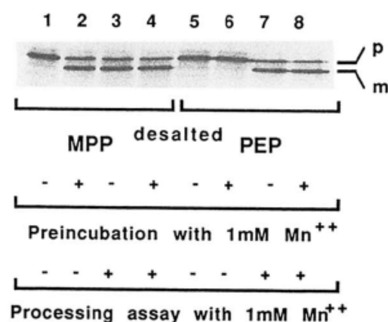

FIG. 7. **Requirement for Mn^{2+} of MPP for processing activity.** Preparations of purified MPP and PEP were desalted with a Sephadex G-25 column. Then 0.2 μg of each protein was incubated separately in the presence or absence of 1 mM $MnCl_2$ for 7 min at 25 °C. To remove unbound manganese ions, MPP and PEP were immunoprecipitated with specific antibodies. Processing activity of the resulting pellet was tested by adding either PEP or MPP and $F_1\beta$ precursor as a substrate. *p*, precursor; *m*, mature $F_1\beta$.

1 and *3*). When Mn^{2+} was included in the enzyme assay all samples showed processing activity (*lanes 5–8*). These results indicate that binding of metal ions, which is necessary for processing activity, occurs to MPP and not to PEP.

DISCUSSION

The amino acid sequence of *Neurospora* mitochondrial processing peptidase, the catalytic component of the matrix processing enzyme, shows a number of striking similarities to that of the yeast counterpart (Pollock *et al.*, 1988; Jensen and Yaffe, 1988). The sequence also shows similarities to that of the other component of the *Neurospora* matrix processing enzyme, the processing enhancing protein (Hawlitschek *et al.*, 1988) which in *Neurospora* is identical to core protein 1 of the respiratory chain complex cytochrome *c* reductase (Schulte *et al.*, 1989). Thus, *Neurospora* MPP appears to be a member of the MPP/PEP/core family.

Unlike yeast MPP, the *Neurospora* MPP has an unusual serine-rich stretch in the center which is not present in any other member of the MPP/PEP/core family. In yeast MPP, however, an exceptionally high number of proline residues are located in this area. We propose that the MPP molecule consists of two domains which are separated by a spacer or a hinge formed by this serine-rich stretch or several proline turns. When one postulates that MPP should have two functions, namely (i) interaction with PEP and (ii) cleavage of presequences, these may be located in the different domains. This would resemble the situation with *Neurospora* PEP/core1 where the amino-terminal half is more similar to yeast PEP while the carboxyl-terminal half is more similar to yeast core1 (Schulte *et al.*, 1989).

The observation of 4 conserved cysteine residues in *Neurospora* and yeast MPP led us to ask whether these are essential for processing activity. Inhibition experiments with sulfhydryl reagents yielded two interesting results. First, inhibition of processing activity occurs with all sulfhydryl reagents employed, the hydrophobic *N*-ethylmaleimide, the hydrophilic iodoacetamide and iodoacetate, and *p*-chloromercuric benzoate. Second, selective treatment of MPP leads to loss of processing activity, but treatment of PEP does not inactivate processing activity.

Specific inhibitors of cysteine proteases did not inhibit the processing activity. Thus, the NEM-sensitive cysteine residues appear not to take part directly in the proteolytic step, and the enzyme therefore appears not to be a member of the class of cysteine proteases. Sequence comparisons of MPP and cysteine proteases showed no regions of similarity. The conserved cysteine residues may have a role in determining the conformation of MPP.

The matrix processing peptidase from several sources has been found to depend on divalent metal ions (Böhni *et al.*, 1980; McAda and Douglas, 1982; Miura *et al.*, 1982; Conboy *et al.*, 1982; Schmidt *et al.*, 1984). Mn^{2+} has to be included in processing assays to obtain full activity. Here we describe that it is MPP that requires manganese ions for processing of precursor proteins, and, on the other hand, that the stimulation of the catalytic activity by PEP is independent of metal ions. Is MPP a metalloprotease as would be indicated by these experiments? In general metalloproteases are not NEM-sensitive, and furthermore no significant homologies to this class of proteases have been observed. Thus, it seems possible that in the matrix peptidase metal atoms are necessary for structure and are not directly involved in the catalytic step.

In summary, the processing enzyme does not appear to belong to any of the known and characterized classes of proteases: not to cysteine proteases and metalloproteases, not to serine proteases because of its insensitivity to PMSF, and not to aspartyl proteases, because pepstatin does not inhibit processing (not shown). The catalytic mechanism therefore remains enigmatic.

The amino terminus of the MPP sequence deduced from the cDNA sequence contains a typical matrix targeting signal which is 35 amino acid residues long and contains an abundance of positively charged residues. When *in vitro* synthesized MPP and PEP were imported into mitochondria, processing to the mature-sized species occurred. Processing during import was reduced in the presence of chelating agents, which are known to inhibit matrix protease (Böhni *et al.*, 1980; Schmidt *et al.*, 1984). Mature MPP and PEP could also be generated by *in vitro* processing with the purified peptidase. Thus, the precursors of MPP and PEP are processed by their (combined) own mature counterparts. Obviously, the continuous presence of functional MPP/PEP is a requirement for MPP/PEP biogenesis. This emphasizes a general principle of mitochondrial biogenesis, namely that formation of new mitochondria depends on pre-existing mitochondria.

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