Matrix Processing Peptidase of Mitochondria

STRUCTURE-FUNCTION RELATIONSHIPS

Helmut Schneider, Michael Arretz, Elmar Wachter, and Walter Neupert

From the Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie, Universität München, Goethestrasse 33, D 8000 München 2, Federal Republic of Germany

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 (Böhm et al., 1980; Hult 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 (Böhm et al., 1980; McAdoo and Douglas, 1982; Miura et al., 1982; Conboy et al., 1982; Schmidt et al., 1984). The enzyme was first purified from Neurospora crassa (Hawitschek 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 coreI 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, MIF is the Ms2 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

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

2. A. Horwich, unpublished results.
of *N. crassa* (Kleene et al., 1987) with the cDNA synthesis kit including EcoRI-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 8% acrylamide 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) 2000-2500 bp, and (e) >2500 bp were obtained following electrophoresis in the cDNAs. The isolated fragments were ligated with 2 μg (a and b) or 1 μg (c-e) of 5'-32P end-labelled *Agtl1-EcoRI* 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) 1.5·10⁴, (b) 5·10⁴, (c) 9·10⁴, (d) 2.8·10⁴, (e) 8·10⁴. The library of (b) was used for screening in the presence of radiolabeled amino acid (Promega). Precursor proteins were synthesized in rabbit reticulocyte lysate containing radiolabeled amino acid and cloning the fragment of the 5'-end into a pGEM3-vector, which was subsequently used for screening for full-length clones. Screening for full-length clones was done by plaque hybridization using the 32P-labeled insert of the antibody-positive clone pMw2 as a probe. 5·10⁴ pfu were grown on five agar plates (94 mm) in Y1088 at 37°C overnight. The plaque DNA was isolated and sequenced by using MPP-specific synthetic oligonucleotide primers, which were designed 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 125I (Perbio-science Sigma; Tsung et al., 1983). Positive clones were picked and rescreened at a density of 220 plaques/94-mm plate. In a second rescreeen we tested them for homogeneity. λDNA was isolated in small scale preparations and cleaved with EcoRI. The cDNA inserts were subcloned into the EcoRI site of pGEM3.

Screening Procedure and Sequencing Strategy—For immuno-screening 5·10⁴ pfu in Y1080 were grown in top agarose (1% Bacto-agar, 0.625% yeast-extract, 0.1 M NaCl, 10 mM MgSO₄, 0.7% agarose) on agar plates with a diameter of 140 mm. In total 2·10⁴ 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 125I (Sigma; Tsung et al., 1983). Positive clones were picked and rescreened at a density of 220 plaques/94-mm plate. In a second rescreeen we tested them for homogeneity. λDNA was isolated in small scale preparations and cleaved with EcoRI. The cDNA inserts were subcloned into the EcoRI site of pGEM3.

Screening for full-length clones was done by plaque hybridization using the 32P-labeled insert of the antibody-positive clone pMw2 as a probe. 5·10⁴ pfu were grown on five agar plates (94 mm) in Y1088 at 37°C overnight. The plaque DNA was fixed in situ on Nynlon membranes (Amerham 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.

Supercoiling sequencing (Chen and Ssehsug, 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 (HindII, HindIII, Sall, Smal) 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 cloned in pGEM (pMPP160) was constructed by cleaving pMk2 with EcoRI and HindIII, blunt-end formation with Klenow enzyme, and cloning the fragment of the 5'-end into a pGEM3-vector, which has been digested with HindIII and XbaI and blunt-ended with Klenow enzyme. By this way the TAG codon in the XbaI-site was replaced by an ATG. The cDNA clones were sequenced and verified by subcloning of fragments into pGEM3. Parts of the cDNA and genomic clones were sequenced by using MPP-specific synthetic oligonucleotide primers.

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 (pH 6.8) and 10 mM PMSF (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) and reisolated on a sodium phosphate gradient. MPP eluted at a phosphate concentration corresponding to 0.1 M PEP. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

After reisolation, mitochondria were incubated in 100 μl SEM containing 4 μg of proteinase K for 25 min at 0°C (Furner 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).

Radioisotope—In Vitro—pMPP160 was synthesized in reticulocyte lysate containing 100 μCi of [14C]methionine, 20 μCi of [14C]glutamic acid, and [3H]valine. Mature sized MPP160 (mPMP160) 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 peptidase (250 μg of protein), and solubilization in SDS containing 1% SDS. The translated polypeptide was cloned into mPMP160 three times in 300 μl of lysis buffer at 22°C for 1 h. Samples containing 25,000 cpm (14C]methionine), 30,000 cpm ([3H]glutamic acid), 18,000 cpm ([3H]valine, imported), and 60,000 cpm ([3H]valine, in vitro processed) were analyzed by solid-phase Edman degradation as described previously (Wachter et al., 1973).

**PURIFICATION OF PROTEIN PEPTIDASE—FURTHER IMPROVEMENT OF PEP**—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 Hawitseh 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 (Hawitseh et al., 1988). The resulting fractions from the PEI-cellulose chromatography containing MPP were then applied to a hydroxyapatite column (BioRad; 2.5 x 10 cm) and chromatographed with a linear 0–200 mM sodium phosphate gradient. MPP eluted at a phosphate concentration of 180–180 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 PFP 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 pGEM3, including the proposed start codon, and lacked only a few bases at the 3' end. pMk2 most likely represented a full-length clone. A genomic DNA-library in pBR322 was also screened by using an antibody probe against Neurospora MPP. We examined 2.5·10⁴ 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 32P-labeled insert of pMw2. Two further clones (pMk1, pMk2) were analyzed, and subcloned into pGFP3. pmk1 started 198 bp downstream of the 5'-end of pMw2 and included a fragment of a poly(A) tail containing 3 adenosine 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 (GGAGCCCATGC versus GGCAGCATGTC versus GGCACCACTGG (Kozak, 1984)).
The open reading frame is indicated by the filled bar in the central line.

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, which has no counterpart in the yeast protein, consists of 265 residues and includes a putative polyadenylation signal (AATARATA) 24 bp upstream of the poly(A) tail. The exact start of the tail was determined by sequencing of a genomic clone.

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

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.

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

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 and to the branch site consensus (light box).
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 metallopro- teases (Kamphuis et al., 1985; Jongeneel et al., 1989). The only similarity we found was to a well-conserved sequence in cysteine proteases (Kamphuis et al., 1985; Jongeneel et al., 1989). The Tyr in stem bromelain (Go10 et al., 1980) is similar to the residues (Cys-126, -207, -416, -435) in MPP of *Neurospora*.

### 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 "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,D. 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 MnCl2 and 1 mM PMSF for 30 min at 25 °C. Samples were analyzed by SDS-gel electrophoresis and fluorography.

Radiosequencing data on the processed form got from import into mitochondria are shown in Fig. 5A. After labeling with "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 epsilon amino groups of lysine residues, every lysine gives a peak. Labeling with "H-valine resulted in two peaks at position 8 (Val-8) and position 11 (Lys-11). When using "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 "H-valine and processed by the purified enzyme was analyzed, peaks at positions 8, 11, 26, and 28 were observed (Fig. 5B). 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-Val-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
mitochondrial precursor proteins, a mitochondrial extract was treated with the sulphydryl reagent N-ethylmaleimide (NEM). As shown in Fig. 5, 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 sulphydryl 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 Mn²⁺ (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 MnCl₂. Then immunoprecipitation with antibodies against either MPP or PEP was carried out in the absence or presence of 1 mM MnCl₂. 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 Mn²⁺ to the assay system, processing was only observed if MPP had been pretreated with Mn²⁺, but not if PEP had been pretreated with Mn²⁺ (Fig. 7, lanes 2 and 4). If Mn²⁺ was absent during pretreatment of MPP and PEP, processing activity was not observed (lanes

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 the mature 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 [³⁵S]methionine (a), [⁴[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 [³⁵S]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.

![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 [³⁵S]methionine (a), [⁴[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 [³⁵S]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.](image)

![Fig. 6. Inactivation of MPP by NEM. Lysed mitochondria (0.5 mg 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 F₁β as a substrate. p, precursor; m, mature F₁β.](image)
precursor as a substrate. p, precursor; m, mature F, fl.

immunoprecipitated with specific antibodies. Processing activity of
consists of two domains which are separated by a spacer or a

tions, namely (i) interaction with PEP and (ii) cleavage of
turns. When one postulates that MPP should have two func-

ions, PEP, while the carboxyl-terminal half is more similar to yeast

sequence contains a typical matrix targeting signal
which is 35 amino acid residues long and contains an abun-
dance of positively charged residues. When in vitro synthe-
sized MPP and PEP were imported into mitochondria, proc-
sessing to the mature-sized species occurred. Processing during
import was reduced in the presence of chelating agents, which
are known to inhibit matrix protease (Böhnì et al., 1980;
Schmidt et al., 1984). Mature MPP and PEP could also be

generated in in vitro processing with the purified peptidase.
Thus, the precursors of MPP and PEP are processed by their
(combined) own mature counterparts. Obviously, the contin-

uous 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 mi-
tochondria depends on pre-existing mitochondria.

Acknowledgments—We thank Drs I. Assfalg-Machleidt and W.
Machleidt for the generous gift of chicken cystatin and E-64. J.
Thierauck kindly helped us in computer analysis. We are grateful
to Drs. M. Tropschug, F.-U. Hartl, and R. A. Stuart for stimulating
discussions and critical reading of the manuscript.

REFERENCES
Attardi, G., and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289-333
Barrett, A. J., Rawlings, N. D., Davies, M. E., Machleidt, W., Sal-
sen, G., and Turk, V. (1986) in (Barrett, A. J., and Salvesen, G.,
eds) Proteinase Inhibitors, pp. 515-599, Elsevier Science Publishing
Co., Amsterdam
Benton, W. D., and Davis, R. W. (1977) Science 196, 180-182
Blake, M. S., Johnston, K. H., Russell-Jones, G. J., and Gatchlick,
E. C. (1984) Anal. Biochem. 136, 175-179
Böhnì, P., Gasser, S., Leaver, C., and Schatz, G. (1980) in (Kroon,
A. M., and Saccone, C., eds) The Organization and Expression of the
Mitochondrial Genome, pp. 423-433, Elsevier Science Publishing
Co., Amsterdam
Bowman, B. J., Allen, R., Wechsler, M. A., and Bowman, E. J. (1988)
J. Biol. Chem. 263, 14002-14007
Burnette, W. J. (1981) Anal. Biochem. 122, 195-203
Chen, E. J., and Seeburg, P. H. (1985) DNA 4, 165-170
Conboy, J. G., Fenton, W. A., and Rosenberg, L. E. (1982) Biochem.
Biophys. Res. Commun. 105, 1-7
Emr, S. D., Vassarotti, A., Barrett, J., Geller, B. L., Takeda, M., and
Douglas, M. G. (1986) J. Cell. Biol. 102, 521-533
Golo, K., Takahashi, N., and Murachi, T. (1980) Int. J. Pept. Protein
Res. 15, 335-341
Hartl, F.-U., Pfanner, N., Nicholson, D. W., and Neupert, W. (1989)
Biochem. Biophys. Acta 988, 1-45
Matrix Processing Peptidase of Mitochondria

Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U., and Neupert, W. (1988) Cell 53, 795–806
Henikoff, S. (1984) Gene (Amst.) 28, 351–359
Hennig, B., and Neupert, W. (1983) Methods Enzymol. 97, 261–274
Horwich, A. L., Kalousek, F., Mallman, I., and Rosenberg, L. E. (1985) EMBO J. 4, 1129–1135
Horwich, A. L., Kalousek, F., Fenton, W. A., Pollock, R. A., and Rosenberg, L. E. (1988) Cell 44, 451–459
Hurt, E. C., Pesold-Hurt, B., and Schatz, G. (1984) FEBS Lett. 178, 306–310
Jensen, R. E., and Yaffe, M. P. (1989) EMBO J. 7, 3863–3871
Jongeneel, C. V., Bouvier, J., and Baircho, A. (1989) FEBS Lett. 242, 211–214
Kalousek, F., Hendrick, J. P., and Rosenberg, L. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7536–7540
Kamphuis, I. G., Dreuth, J., and Baker, E. N. (1985) J. Biol. Chem. 182, 317–329
Keng, T., Alani, E., and Guarente, L. (1986) Mol. Cell. Biol. 6, 355–364
Kleene, R., Pfanner, N., Pfaller, R., Link, T. A., Sebald, W., Neupert, W., and Tropschug, M. (1987) EMBO J. 6, 2827–2833
Kozak, M. (1984) Nucleic Acids Res. 12, 857–872
Laemmli, U. K. (1970) Nature 227, 680–685
McAda, P. C., and Douglas, M. G. (1982) J. Biol. Chem. 257, 3177–3182
Miura, S., Mori, M., Amaya, Y., and Tatibana, M. (1982) Eur. J. Biochem. 122, 641–647
Nicholson, D. W., and Neupert, W. (1988) in (Das, R. C., and Robbins, P. W., eds) Protein Transfer and Organelle Biogenesis, pp. 677–746, Academic Press, New York
Ou, W.-J., Ito, A., Okazaki, H., and Omura, T. (1989) EMBO J. 8, 2605–2612
Oudhoorn, P., van Steeg, H., Swinkels, B. W., Schoppenk, P., and Grivell, L. A. (1987) Eur. J. Biochem. 163, 97–103
Pelham, H. R. B., and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247–256
Pfanner, N., and Neupert, W. (1985) EMBO J. 4, 2819–2825
Pollock, R. A., Hartl, F.-U., Cheng, M. Y., Ostermann, J., Horwich, A., and Neupert, W. (1988) EMBO J. 7, 3453–3460
Rich, D. A. (1986) in (Barrett, A. J., and Salvesen, G., eds) Proteinase Inhibitors, pp. 153–178, Elsevier Science Publishing Co., Amsterdam
Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., and Schatz, G. (1986) EMBO J. 5, 1327–1334
Sanger, F., Mcklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. U. S. A. 74, 5463–5467
Schmidt, B., Wachter, E., Sebald, W., and Neupert, W. (1984) Eur. J. Biochem. 144, 581–588
Schulte, U., Arretz, M., Schneider, H., Tropschug, M., Wachter, E., Neupert, W., and Weiss, H. (1989) Nature 339, 147–149
Tsung, V. C. W., Peralta, J. M., and Simons, H. R. (1983) Methods Enzymol. 92, 377–391
Tzagoloff, A., Wu, M., and Crivellone, M. (1986) J. Biol. Chem. 261, 7183–7189
Vassarotti, A., Stroud, R., and Douglas, M. G. (1987) EMBO J. 6, 705–711
Witte, C., Jensen, R. E., Yaffe, M. P., and Schatz, G. (1988) EMBO J. 7, 1439–1447
Yang, M., Jensen, R. E., Yaffe, M. P., Oplizner, W., and Schatz, G. (1988) EMBO J. 7, 3857–3862
Young, R. A., and Davis, R. W. (1983) Science 222, 778–782