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 (Hawliitschek et al., 1988). Two proteins are required for proteolytic activity, the mitochondrial processing peptidase (MPP) 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). 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 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 the catalytic 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 Sliced 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 (Kleine 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% 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-
colation of the cDNA. The isolated fragments were ligated with 2 µg (a and b) or 1 µg (c-e) of Xgt11-EcoRI arms (Pharmacia LKB Biotechnology Inc.), respectively. Immediately after packaging (Giga
gpak Gold, Stratagene) the libraries were amplified in Escherichia coli strain Y1088. The number of different phages before amplification were (a) 1.5 x 10^5, (b) 5 x 10^5, (c) 9 x 10^5, (d) 2.8 x 10^6, (e) 8 x 10^6. The library was then screened, and 9 x 10^6 pfu plaques were screened and genomic clones were isolated. A genomic DNA-library in pBR322 was also screened and genomic clones were sequenced only in part. An outline of the sequenc-
ing strategy is given in Fig. 1. The cDNA consists of 2037 bp and was cleaned by hybridization using the 32P-labeled insert of the antibody-positive clone pMk2 as a probe. 5 x 10^6 pfu were grown on five agar plates (94 mm) in Y1088 at 37 °C overnight. The plaque DNA was fixed in Xgt11 (Young and Davis, 1983) was screened by using an antibody probe containing MPP were then applied to a hydroxyapatite column (Bio-
RAD; 2.5 x 10 cm) and chromotographed with a linear 0-200 mM sodium phosphate gradient. MPPε 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 500 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 Se-
quence of Neurospora MPP—A cDNA library of N. crassa containing cDNA inserts in the range of 1500-2000 bp cloned in Xgt11 (Young and Davis, 1983) was screened by using an antibody probe against Neurospora MPP. We examined 2.5-10^6 plaque 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 pMk2. The cDNA-insert of pMk2 was 1902 bp in length. The library was then rescreened with the 32P-labeled insert of pMk2. Two further clones (pMk1, pMk2) were analyzed and subcloned into pGEM3. pMk1 started 198 bp downstream of the 5'-end of pMk2 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 represents 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 sequenc-
ing 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 (GCGACCATGCG versus GCCACCATGG (Kozak, 1984)). The
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 an 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).

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.

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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).
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 (Hawlitschek et al., 1988; Jensen and Yaffe, 1988). The only similarity we found was to a well-conserved sequence in cysteine proteases. 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 SpG-RNA polymerase followed by *in vitro* translation in rabbit reticulocyte lysate 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. 5A. After labeling with [35S]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 [3H]valine resulted in two peaks at position 8 (Val-8) and position 11 (Lys-11). When using [3H]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 [3H]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-Val-Leu-Ala-Thr-Arg. This cleavage site is in agreement with the consensus Arg-X-Y-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 sulfhydryl reagents iodoacetate 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; Hawliitschek 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 1, 4, and 7).

**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 F$_i$,$\Delta$ as a substrate. p, precursor; m, mature F$_i$,$\Delta$.
precursor as a substrate. p, precursor; m, mature F, fl.

25 °C. To remove unbound manganese ions, MPP and PEP were

tions, namely (i) interaction with PEP and (ii) cleavage of

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 func-

tions, 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/

corel where the amino-terminal half is more similar to yeast

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

corel (Schulte et al., 1989).

The observation of 4 conserved cysteine residues in Neu-

rospora and yeast MPP led us to ask whether these are

essential for processing activity. Inhibition experiments with

sulfhydryl reagents yielded two interesting results. First, in-\n
hibition of processing activity occurs with all sulfhydryl re-

agents employed, the hydrophobic N-ethylmaleimide, the

hydrophilic iodoacetamide and iodoacetate, and p-chloromer-

curic 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 resi-
dues 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 (Böhnì et al.,

1980; McAda and Douglas, 1982; Miura et al., 1982; Conboy

et al., 1982; Schmidt et al., 1984). Mn²⁺ 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 stimula-

tion 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-sen-
sitive, 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 struc-
ture 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-

ized 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 by in vitro processing with the purified peptidase.

Thus, the precursors of MPP and PEP are processed by their

(combined) own mature components. 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.

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Matrix Processing Peptidase of Mitochondria


Matrix processing peptidase of mitochondria. Structure-function relationships.
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