

Cleavage of Precursors by the Mitochondrial Processing Peptidase Requires a Compatible Mature Protein or an Intermediate Octapeptide

Grazia Isaya, Frantisek Kalousek, Wayne A. Fenton, and Leon E. Rosenberg

Yale University School of Medicine, Department of Human Genetics, New Haven, Connecticut 06510

Abstract. Many precursors of mitochondrial proteins are processed in two successive steps by independent matrix peptidases (MPP and MIP), whereas others are cleaved in a single step by MPP alone. To explain this dichotomy, we have constructed deletions of all or part of the octapeptide characteristic of a twice cleaved precursor (human ornithine transcarbamylase [pOTC]), have exchanged leader peptide sequences between once-cleaved (human methylmalonyl-CoA mutase [pMUT]; yeast F_1 ATPase β -subunit [pF $_1\beta$]) and twice-cleaved (pOTC; rat malate dehydrogenase (pMDH); *Neurospora* ubiquinol-cytochrome *c* reductase iron-sulfur subunit [pFe/S]) precursors, and have incubated these proteins with purified MPP and MIP. When the

octapeptide of pOTC was deleted, or when the entire leader peptide of a once-cleaved precursor (pMUT or pF $_1\beta$) was joined to the mature amino terminus of a twice-cleaved precursor (pOTC or pFe/S), no cleavage was produced by either protease. Cleavage of these constructs by MPP was restored by re-inserting as few as two amino-terminal residues of the octapeptide or of the mature amino terminus of a once-cleaved precursor. We conclude that the mature amino terminus of a twice-cleaved precursor is structurally incompatible with cleavage by MPP; such proteins have evolved octapeptides cleaved by MIP to overcome this incompatibility.

THE vast majority of mitochondrial proteins are encoded by nuclear genes and synthesized as larger precursors bearing amino-terminal leader peptides that contain sufficient information to direct mitochondrial localization. During or after translocation of the precursor proteins into the mitochondrial matrix, the leader peptides are proteolytically cleaved to produce mature forms (for reviews see Rosenberg et al., 1987; Verner and Schatz, 1988; Pfanner and Neupert, 1990). Most leader peptides are removed in one step by the mitochondrial processing peptidase (MPP)¹ (Pollock et al., 1988; Jensen and Yaffe, 1988; Schneider et al., 1990; Kleiber et al., 1990) with the cooperation of the processing enhancing protein (PEP) (Hawlit-schek et al., 1988; Witte et al., 1988; Yang et al., 1988; Ou et al., 1989), while others are removed in two sequential steps (Sztul et al., 1987; 1988; Hartl et al., 1986; Tropschug et al., 1988) by two distinct matrix peptidases (Kalousek et al., 1988). These leaders are initially processed to an intermediate form by MPP. Formation of the mature protein is then catalyzed by a mitochondrial intermediate peptidase (MIP) that removes eight amino acids (the octapeptide) from the amino terminus of the intermediate.

Extensive amino acid sequence analysis in the regions surrounding the MPP and MIP cleavage sites has suggested some of the features that may be responsible for recognition and cleavage of the leader peptides by the matrix peptidases (for reviews see von Heijne et al., 1989; Hendrick et al., 1989). An arginine residue is frequently found at position -2 from the MPP cleavage sites, while the octapeptides cleaved by MIP often include a hydrophobic residue at position -8, and serine, threonine, or glycine at position -5, relative to the mature amino terminus. Importantly, a specific primary amino acid sequence has not been identified on the carboxy-terminal side of either MPP or MIP cleavage sites.

These data suggest that the matrix peptidases may recognize a higher order protein structure, rather than specific amino acid residues at the junctions between the leader peptides and the mature amino termini (von Heijne et al., 1989; Hendrick et al., 1989). Further, the lack of correlation between one- or two-step processing of the precursors and the biological function of the corresponding mature proteins implies that two-step processing evolved mainly to respond to specific structural characteristics of a subset of mitochondrial protein precursors. We have postulated that the mature portion of twice-cleaved precursors is structurally incompatible with cleavage by MPP and that the octapeptides function to supply the structural requirements for cleavage (Hendrick et al., 1989).

1. *Abbreviations used in this paper:* MIP, mitochondrial intermediate peptidase; MPP, mitochondrial processing peptidase.

We report here a test of this hypothesis. Novel precursors, derived from once- and twice-cleaved proteins, were generated by deletion, insertion, or exchange of octapeptides or leader peptides; cleavage of these precursors was then tested either with purified MPP and MIP or with isolated rat liver mitochondria.

Materials and Methods

Materials

DEAE Bio-Gel A was from Pharmacia Fine Chemicals (Piscataway, NJ). Centriprep concentrators were from Amicon Corp. (Danvers, MA). Heparin-agarose and ω -amino-octylagarose were from Sigma Chemical Co. (St. Louis, MO). Zn chelate affinity adsorbent was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Oligonucleotides were synthesized by a DNA synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA). Restriction enzymes were from New England Biolabs (Beverly, MA). Rabbit reticulocyte lysate was from Promega Biotec (Madison, WI). NuSieve GTG and SeaPlaque GTG agaroses were from FMC Corp. (Rockland, ME). SP6 and T7 phage RNA polymerases were from Boehringer-Mannheim Biochemicals. [35 S]Methionine was from Amersham Corp. (Arlington Heights, IL). Polyvinylidene difluoride (PVDF) membrane was from Millipore Corp. (Bedford, MA).

Preparation of Mitochondrial Matrix Peptidases

Preparation of Mitochondrial Matrix. Mitochondrial matrix was prepared as previously described (Conboy et al., 1982) except that in these experiments, livers from 20 rats (male, Sprague-Dawley, 100–120 g) were used. The yield was usually between 1,000 and 1,300 mg of total protein.

DEAE Bio-Gel A Chromatography. In a typical experiment, the mitochondrial matrix fraction from 20 rats was diluted to 180 ml with 10 mM Hepes, pH 7.4, 0.5 mM DTT, containing NaCl to a final concentration of 20 mM, and loaded on a DEAE Bio-Gel A column (2.5 \times 6 cm), equilibrated with the same buffer, at a flow rate of 60 ml/h. After adsorption, the column was washed with two column volumes of the same buffer, followed by 50 mM NaCl, 10 mM Hepes, pH 7.4, 0.5 mM DTT (30 column volumes at a flow rate of 120 ml/h, or until the absorbance at 280 nm of the flow through was < 0.1). Both MPP and MIP were then eluted with 100 ml of 100 mM NaCl, 10 mM Hepes, pH 7.4, 0.5 mM DTT.

Heparin Agarose I. The eluate from DEAE Bio-Gel A column was concentrated on a Centriprep 10 concentrator to 20 ml, then diluted with 10 mM Hepes, pH 7.4, 0.5 mM DTT to a final concentration of 30 mM NaCl. The sample was loaded on a heparin-agarose column (2.5 \times 6 cm), equilibrated with the same buffer, at a flow rate of 18 ml/h. Both enzymes were eluted in the flow-through with 40 ml of the same buffer.

Hydroxyapatite Chromatography. The Heparin-agarose I fraction was diluted with 10 mM Hepes, pH 7.4, 0.5 mM DTT to a final concentration of 20 mM NaCl and loaded on a hydroxyapatite column (1.5 \times 1.5 cm), equilibrated with the same buffer, at a flow rate of 18 ml/h. After adsorption, the column was washed with 10 mM potassium phosphate buffer, pH 7.0, 0.5 mM DTT. About 30% of the total MPP activity was eluted but not used for further purification because of the low specific activity. The major portion of MPP and all of MIP activity were then eluted by a linear gradient from 10 mM potassium phosphate, pH 7.0, 0.5 mM DTT to 60 mM potassium phosphate, pH 7.8, 0.5 mM DTT.

ω -Aminoocetyl-Agarose. The active fractions were pooled and loaded directly on a ω -aminoocetyl-agarose column (1 \times 2.5 cm) equilibrated with 10 mM Hepes, pH 7.4, 0.5 mM DTT at a flow rate of 18 ml/h. The column was washed with 10 column volumes of 100 mM NaCl, 10 mM Hepes, pH 7.4, 0.5 mM DTT, then eluted stepwise with 150 mM, 200 mM, 300 mM, and 600 mM NaCl in the same buffer (6 ml portions). Approximately 80% of MPP activity was found in the 200 and 300 mM NaCl eluates, the remaining 20% was eluted at 600 mM NaCl; 20% of MIP activity was eluted at 300 mM NaCl, and the remaining 80% was recovered in the 600 mM NaCl fraction.

Heparin-Agarose II. The 200 and 300 mM NaCl eluates were pooled, concentrated on a Centricon 30 microconcentrator, diluted to 20 mM NaCl in 10 mM Hepes, pH 7.4, 0.5 mM DTT and reconcentrated to a final volume of 3 ml. The sample was loaded on a heparin-agarose column (0.6 \times 1 cm) equilibrated with the same buffer. The column was washed with 3 column vol of the same buffer. This flowthrough eluate contained only MPP activity.

MIP free of MPP was then eluted with 75 mM NaCl, 10 mM Hepes, pH 7.4, 0.5 mM DTT.

Zn Chelate. The Zn chelate affinity adsorbent column was loaded with Zn acetate, pH 5.5, as recommended by the manufacturer. After extensive washing with distilled H₂O, the column (0.6 \times 1 cm) was equilibrated with 50 mM Tris-PO₄, pH 7.4 and the heparin-agarose II eluate with MPP activity was loaded. The column was washed with 10 column vol of the same buffer, followed by 10 column vol of 100 mM NaCl in 10 mM potassium phosphate buffer, pH 6.4, 0.5 mM DTT. MPP activity was eluted with 5 column vol of 800 mM NaCl in the same buffer. The protein was concentrated, diluted in 10 mM Hepes, pH 7.4, 0.5 mM DTT, reconcentrated to a final volume of 100 μ l. This preparation has been stored at -70°C without any loss of activity for at least 6 mo.

In Vitro Transcription and Translation

Messenger RNAs were produced by in vitro transcription from the various wild-type and mutant cDNAs cloned in transcription vectors. Transcriptions with phage SP6 and T7 polymerases and in vitro translation using rabbit reticulocyte lysates were performed according to supplier recommendations.

Incubation of the Translation Mixtures with Intact Mitochondria and with Purified Processing Peptidases

Intact mitochondria from rat liver were prepared as described (Conboy et al., 1981). 6 μ l of translation product were incubated with 4 μ l of a suspension of freshly isolated rat liver mitochondria (20 mg/ml) for 20 min at 27°C. After the import reaction, aliquots were treated with trypsin (final concentration 40 $\mu\text{g/ml}$) for 10 min on ice and then supplemented with soy-

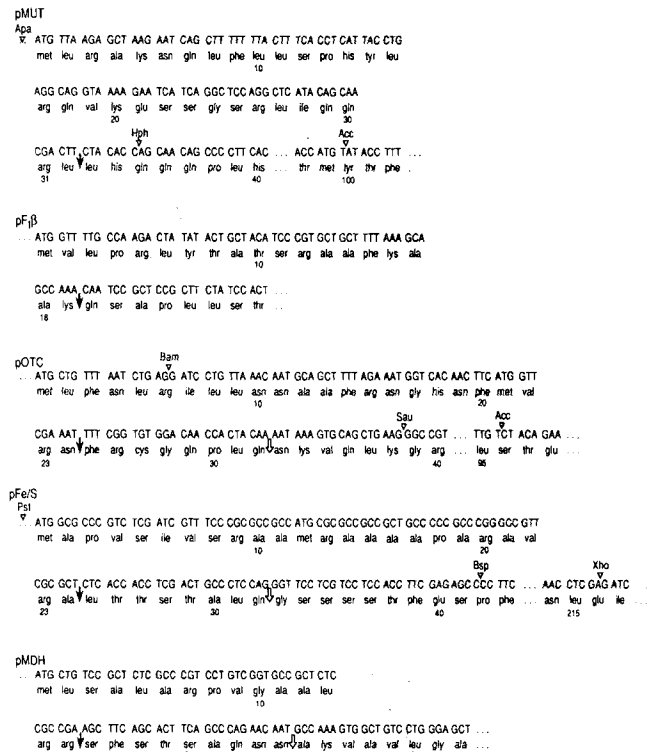


Figure 1. Nucleotide and primary amino acid sequences of the leader peptides and mature amino termini of the once- and twice-cleaved precursors used. Normal sites of MPP and MIP cleavage of the leader peptides are designated by solid and open arrows, respectively; triangles denote restriction endonuclease cutting sites. Abbreviations used: Bam, Bam HI; Sau, Sau 96 I; Acc, Acc I; Apa, Apa I; Hph, Hph I; Pst, Pst I; Bsp, Bsp 1286 I; Xho, Xho I.

bean trypsin inhibitor at a final concentration of 100 $\mu\text{g/ml}$. Products of the import reactions were analyzed directly by SDS/PAGE.

Mitochondrial processing peptidase fractions were diluted to the required concentration with 10 mM Hepes, pH 7.4, 0.5 mM DTT containing 0.1 mg/ml BSA. In a typical experiment, 1 μl of translation mixture was supplemented with 2.5 μl of 100,000 g supernatant from reticulocyte lysate and incubated with the MPP fraction or the MIP fraction in a final volume of 10 μl for 1 h at 27°C. All reactions contained 0.1 mM ZnCl_2 and 0.1 mM MnCl_2 . Products of the reactions were analyzed directly on SDS/PAGE.

Mutagenesis

Fig. 1 shows the nucleotide and primary amino acid sequences of the leader peptides and mature amino termini of the mitochondrial precursors used as models in the present study. Plasmid pSPOTC, containing the SP6 promoter joined with the human OTC cDNA, and plasmid pGEMMUT, containing the T7 promoter joined to the human MUT cDNA, have been described before (Horwich et al., 1984; Jansen et al., 1989). Plasmids containing the rat pMDH cDNA (Grant et al., 1986; Chu et al., 1987), the yeast $\text{pF}_1\beta$ cDNA (Takeda et al., 1985, 1986), and the *Neurospora* pFe/S cDNA (Harnish et al., 1985), joined to the SP6 promoter, were kindly provided by Paula Grant and Arnold Strauss, from the Washington University (St. Louis, MO), David Bedwell and Scott Emr, from the California Institute of Technology (Pasadena, CA), and by Franz-Ulrich Hartl and Walter Neupert, from Munich University (Munich, Germany), respectively.

We introduced specific deletions and substitutions in the wild-type pOTC and pMUT cDNAs (see Fig. 1) by polymerase chain reaction (PCR) amplification (Mullis et al., 1989). For construction of pOTC molecules containing serial deletions in the octapeptide, we designed a primer complementary to the DNA sequence coding for the amino terminus of the pOTC leader peptide and four antisense primers noncomplementary to the sequences coding for residues 25–32, 27–32, 29–32, and 31–32, respectively, that hybridized to flanking sequences in the leader peptide and in the mature amino terminus. In the case of deletion 31–32, the proline residue at position 30 was also changed to a glutamine to exclude the possibility that proline could interfere with cleavage at the expected site. Using the wild-type pOTC cDNA as template sequence, we amplified four fragments containing the desired deletions in the octapeptide coding sequence from a unique Bam HI site in the leader peptide sequence to a convenient Sau 96 I site in the mature amino terminus. For subcloning purposes, we also amplified a cDNA fragment from the Sau 96 I site to a unique Acc I site in the mature coding sequence. PCR amplification products were digested with Bam HI and Sau 96 I or Acc I and Sau 96 I, as required, and purified through a 3% NuSieve GTG low melting agarose gel. Plasmid pSPOTC was digested with Bam HI and Acc I to provide the bulk of the coding sequence and plasmid backbone and purified through an 0.8% SeaPlaque GTG low-melting agarose gel. Gel slices containing the bands corresponding to the inserts and the linearized plasmid were recovered, remelted at 65°C for 10 min, and used directly in the ligation reactions. After bacterial transformations, the desired recombinants were identified by restriction analysis. All of the mutational alterations were verified by DNA sequence analysis.

Construction of pOTC molecules containing either an artificial octapeptide (OTC-(art8)-OTC) or the MDH (residues 17–24) (OTC-(MDH8)-OTC) or Fe/S (residues 25–32) (OTC-(Fe/S8)-OTC) octapeptide was carried out essentially as described above, using an antisense primer complementary to the DNA sequence coding for the mature amino terminus at the 5' end,

for residues 20–24 of the leader peptide at the 3' end, and for a noncomplementary DNA sequence coding for the desired octapeptide in between.

Construction of an iOTC molecule bearing an initiating methionine, instead of phenylalanine at position –8 from the mature OTC amino terminus (Met-iOTC), was carried out using a primer complementary to the DNA sequence coding for residues 26–32 of the pOTC octapeptide with a noncomplementary extension at the 5' end containing a Bam HI site, a stop codon, a consensus sequence (Kozak, 1987) and an ATG codon.

For construction of the MUT-OTC chimera, we designed a primer complementary to the DNA sequence coding for the amino terminus of pMUT, carrying a noncomplementary extension on the 5' end that contained a Bam HI site, a stop codon, and a consensus Kozak sequence. The corresponding antisense primers were complementary to the DNA sequences coding for the carboxy terminus of the pMUT leader peptide and, when required, for the 2, 6, and 8 amino-terminal residues of mature MUT, respectively. At the 5' end, they carried a noncomplementary extension containing the DNA sequence coding for the amino terminus of mature OTC, including a Sau 96 I restriction site. Using the wild-type pMUT cDNA as template sequence, we synthesized four specific DNA fragments containing the entire coding sequence for the pMUT leader peptide joined to the amino terminus of mature OTC, either directly (MUT-OTC) or through the two (MUT2-OTC), six (MUT6-OTC), or eight (MUT8-OTC) amino-terminal residues of mature MUT. In the case of MUT6-OTC, the proline residue at position –1 relative to the mature OTC amino terminus was changed to glutamine. These fragments were digested with Bam HI and Sau 96 I and subcloned into a Bam HI-Acc I-digested plasmid pSPOTC as described above.

For construction of the chimera carrying the 24 amino-terminal residues of the pOTC leader peptide joined to the mature MUT amino terminus (OTC (1-24)-MUT), we designed a primer complementary to the cDNA sequence coding for the amino terminus of the pOTC leader peptide, carrying a noncomplementary extension on the 5' end that contained an Apa I site and a consensus Kozak sequence. The corresponding antisense primer was complementary to the DNA sequences coding for residues 20–24 (not including the octapeptide) of the pOTC leader peptide carboxy terminus and carried a noncomplementary extension at the 5' end containing the DNA sequence coding for the mature MUT amino terminus, including a convenient Hph I site. For subcloning purposes, we also amplified a fragment of the pMUT cDNA from an Hph I site to a unique Acc I site in the mature coding sequence. PCR products were digested with Apa I and Hph I, or with Acc I and Hph I, as required; plasmid pGEMMUT was digested with Apa I and Acc I. The small inserts and linearized vector were purified in low melting agarose gels and ligated as described above.

For construction of the $\text{F}_1\beta$ -Fe/S chimera we designed a primer complementary to the DNA sequence coding for the amino terminus of $\text{pF}_1\beta$, carrying a noncomplementary extension on the 5' end that contained a Pst I site and a Kozak sequence. The corresponding antisense primer was complementary to the DNA sequence coding for the carboxy terminus of the $\text{pF}_1\beta$ leader peptide, and carried a noncomplementary extension on the 5' end that contained the DNA sequence coding for the amino terminus of mature Fe/S, including a convenient Bsp 1286 I site. Using the $\text{pF}_1\beta$ cDNA as template sequence, we amplified a DNA fragment containing the entire coding sequence for the $\text{pF}_1\beta$ leader peptide joined directly to the amino terminus of mature Fe/S. For subcloning purposes, we also amplified the DNA fragment from the Bsp 1286 I site to a unique Xho I site in the mature Fe/S coding sequence. The PCR amplification products were subcloned

Table I. Purification of Mitochondrial Processing Peptidase from Rat Liver Mitochondria

Fraction	Total protein	Total activity	Specific activity	Purification factor	Yield
	mg	$U \times 10^{-3}$	U/mg		%
Matrix	1,160	360	310	1.0	100
DEAE Bio-Gel A	109	165	1,513	4.8	46
Heparin-agarose I	16	154	9,625	31.0	43
Hydroxyapatite	4.5	72	16,000	61.0	20
ω -Aminooctyl-Sepharose	0.4	50	125,000	403	14
Heparin-agarose II	0.12	39	325,000	1,048	11
Zn chelate	0.034	16	490,000	1,580	4.6

One unit of activity is defined as the amount of enzyme that catalyzes the conversion of 50% of the pMUT in 1 μl of in vitro translation mixture to its mature form in 15 min at 27°C.

into the large fragment of a Pst I-Xho I-digested plasmid pGEM3-Fe/S, as described above.

Amino Acid Sequence Analysis

Radiolabeled precursors were synthesized in a rabbit reticulocyte lysate containing 1 mCi of [³H]leucine. Translation mixtures were then incubated with the MPP or the MIP fraction as required, under the conditions described above. The products of the processing reaction were separated on SDS/PAGE and electroblotted onto a PVDF membrane. The bands corresponding to the products of interest were localized, excised, and sequenced directly on a sequencer (model 470A; Applied Biosystems, Inc.) as described (Matsudaira, 1987).

Results

Purification and Characterization of MPP and MIP from Rat Liver Mitochondria

We showed previously that partially purified matrix fractions of rat liver mitochondria contain two distinct processing activities (Kalousek et al., 1988). The first, MPP (previously called P₁ [Kalousek et al., 1988] or MPP-I [Kleiber et al., 1990]) was shown to cleave the precursor for the β -subunit of rat propionyl-CoA carboxylase to its mature-sized form, and the precursors for rat OTC and rat MDH to their intermediate sized forms (iOTC and iMDH). The second, MIP (previously called P₂ [Kalousek et al., 1988] or MPP-II [Kleiber et al., 1990]) was shown to cleave iOTC and iMDH to their respective mature-sized forms. We have now separated these two activities and purified MPP to homogeneity.

We purified MPP as described in Materials and Methods and a typical result is summarized in Table I. With a seven-

step procedure, we achieved nearly 1,600-fold purification, with a final yield of ~5%. The various purification steps were monitored by SDS/PAGE and staining of protein bands with Coomassie blue (Fig. 2). At the end of the first five steps of purification (Fig. 2, lane 6), we obtained a fraction with MPP activity containing three major bands of 55, 52, and 42 kD on SDS-PAGE. The 42-kD protein could be removed by Zn-chelate adsorption, without affecting the processing activity of MPP. The final fraction with MPP activity (lane 7) contained only the 55- and 52-kD proteins, which were not separated from each other at any step in the purification. These findings are very similar to those obtained previously by Ou et al. (1989), using a similar approach and very similar chromatographic steps. These authors did not comment on the presence of MIP activity in their fractions. We observed MIP activity copurifying with MPP in the first four steps of the purification. At step 5, using ω -aminooctyl agarose followed by heparin-agarose chromatography, we separated MPP from MIP, without affecting the processing activity of either protease.

Antiserum raised against a partially purified fraction containing the 55-, 52-, and 42-kD proteins (Fig. 2, lane 6; Kleiber et al., 1990) immunoprecipitated the MPP activity from a crude matrix fraction and from a purified MPP fraction (data not shown). This antiserum also recognized the 55-kD protein on Western blots after SDS/PAGE of rat liver mitochondrial matrix (not shown).

The enzymatic properties of the MPP and MIP fractions were analyzed by incubation with several mitochondrial precursors whose patterns of processing had been previously determined with isolated mitochondria or intact cells. The

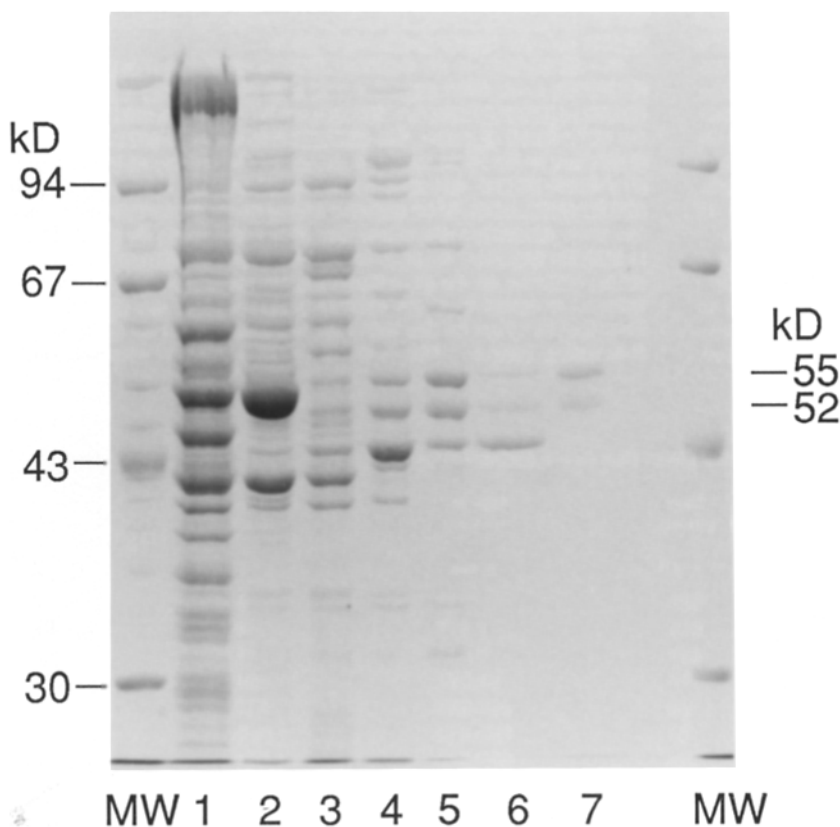


Figure 2. Purification of MPP as analyzed by SDS-PAGE. 20- μ l aliquots of different fractions were electrophoresed on SDS/PAGE (8%). Pharmacia electrophoresis calibration kit was used for protein standards. Proteins were stained with Coomassie brilliant blue R-250. Lane 1, mitochondrial matrix (100 μ g); lane 2, DEAE Bio-Gel eluate (60 μ g); lane 3, heparin-agarose I fraction (30 μ g); lane 4, hydroxyapatite pool (10 μ g); lane 5, ω -aminooctyl-agarose eluate (7 μ g); lane 6, heparin-agarose II fraction (1.3 μ g); lane 7, Zn chelate eluate (0.7 μ g); MW, molecular weight marker proteins (0.3–0.7 μ g) as specified by the supplier.

precursor proteins were synthesized in a rabbit reticulocyte lysate and then incubated with MPP and/or MIP, under the conditions described in Materials and Methods. The precursors for human MUT and yeast $F_1\beta$, which are normally cleaved in one step (Fenton et al., 1984; Vassarotti et al., 1987), were processed to their mature-sized forms by MPP (Fig. 3 a, lanes 2 and 6). No proteolytic activity was observed upon incubation of pMUT and p $F_1\beta$ with MIP (lanes 3 and 7). Incubation with MPP plus MIP did not have any additional effect as compared to incubation with MPP alone (lanes 4 and 8). Radiosequence analysis of the mature MUT (Fig. 3 b) indicated that purified MPP had generated the same mature amino terminus as was previously determined by amino acid sequence analysis of purified human liver MUT (Jansen et al., 1989).

The precursors for *Neurospora* Fe/S, rat MDH, and human OTC, normally cleaved in two sequential steps (Hartl

et al., 1986; Sztul et al., 1987, 1988), were processed to their respective intermediate-sized forms by MPP (Fig. 3 a, lanes 10, 14, and 18). Radiosequence analysis of iFe/S (Fig. 3 c) indicated that MPP had generated the same intermediate amino terminus as was previously determined by radiosequence analysis of iFe/S generated by purified *Neurospora* MPP (Hartl et al., 1986). Incubation of these precursors with MPP plus MIP (lanes 12, 16, and 20) led to conversion of the intermediate species to their respective mature forms. Radiosequence analysis of mature OTC (Fig. 3 d) indicated that sequential cleavage of pOTC by MPP and MIP had generated the same amino terminus as was previously determined by amino acid sequence analysis of purified human liver OTC (Horwich et al., 1984). No proteolytic activity was observed upon incubation of pFe/S, pMDH, and pOTC with MIP (Fig. 3 a, lanes 11, 15, and 19), consistent with the idea that formation of the intermediates by MPP is required

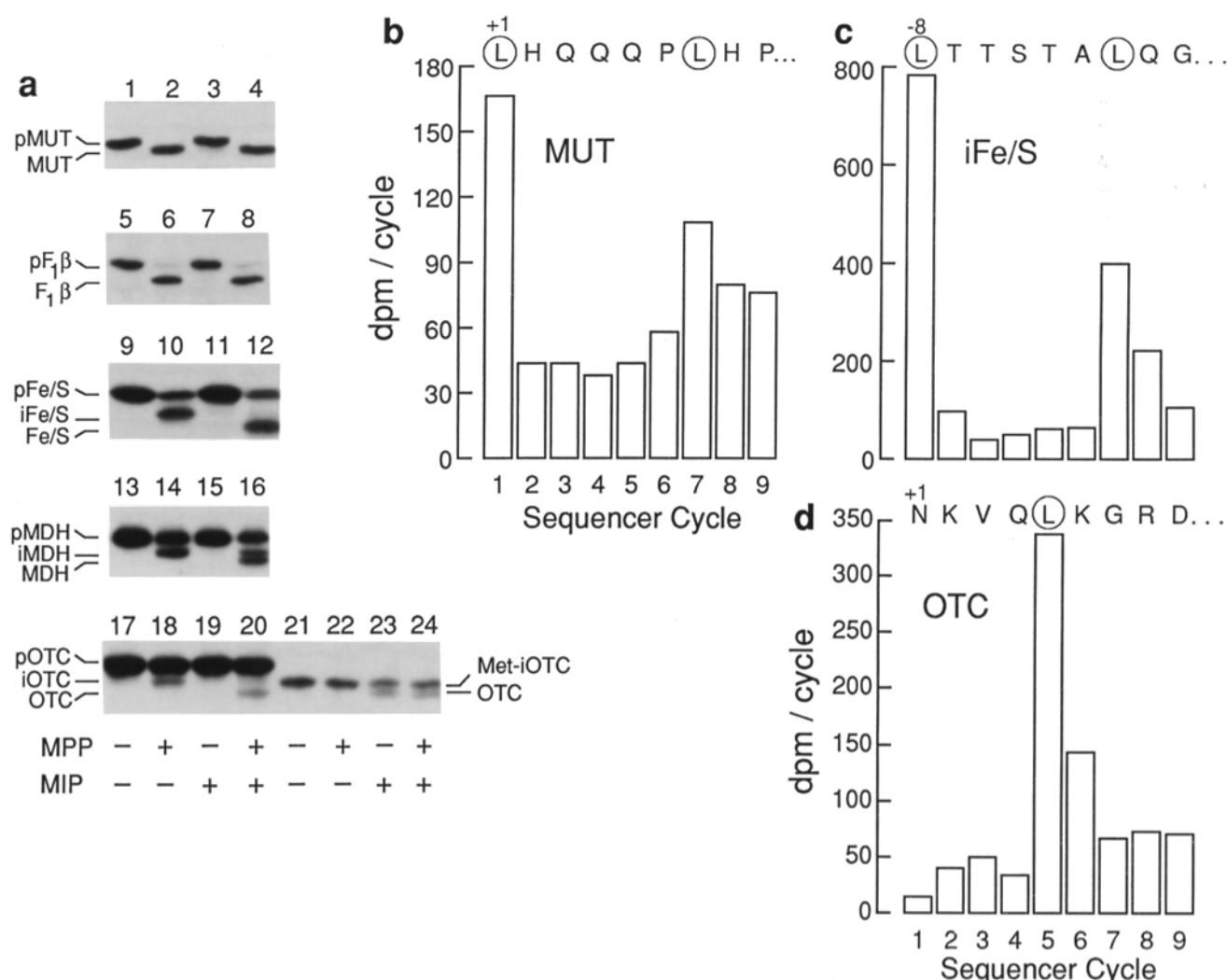


Figure 3. One- and two-step processing of mitochondrial precursors by MPP and MIP. (a). [35 S]Methionine-labeled precursors were incubated with MPP (lanes 2, 6, 10, 14, 18, and 22), MIP (lanes 3, 7, 11, 15, 19, and 23), and MPP plus MIP (lanes 4, 8, 12, 16, 20, and 24) for 1 h at 27°C as described in Materials and Methods. The products of the processing reactions were analyzed directly on SDS-PAGE. Met-iOTC indicates an iOTC molecule bearing an initiating methionine instead of phenylalanine at -8 from the mature OTC amino terminus. (b-d) Wild-type pMUT, pFe/S, and pOTC were synthesized in the presence of [3 H]leucine and incubated with MPP (pMUT and pFe/S) or MPP plus MIP (pOTC) for 1 h at 27°C. Mature MUT (b), iFe/S (c), and mature OTC (d) were subjected to radiosequencing as described in Materials and Methods. The amino acid sequences of mature MUT, iFe/S, and mature OTC are shown; +1 marks the amino termini of mature MUT and mature OTC; -8 marks the amino terminus of iFe/S; labeled leucines are indicated by circled residues.

for subsequent formation of the mature proteins by MIP (Sztul et al., 1987). This result is also consistent, however, with the possibility that MIP is not an independent protease, but a cofactor required by MPP to complete maturation of twice-cleaved precursors. To distinguish between these two possibilities, we synthesized an iOTC molecule bearing an initiating methionine, instead of phenylalanine, at position -8 from the mature OTC amino terminus (Met-iOTC). This substrate allowed us to test the proteolytic properties of the MIP fraction independent of the presence of MPP. Met-iOTC was processed by MIP to mature OTC (Fig. 3 a, lane 23) and incubation with MIP plus MPP did not have any additional effect (lane 24). No proteolytic activity was observed upon incubation of Met-iOTC with MPP alone (lane 22).

These results lend strong support to the view that MPP and MIP are the components that cleave imported precursors in vivo. Therefore, we used these two peptidase fractions to analyze the structural requirements of mitochondrial presequences for proteolytic processing.

Deletion of the Octapeptide Blocks Processing of pOTC

To analyze the role of the octapeptide found at the carboxy terminus of the 32-amino acid pOTC leader in mediating two-step processing of pOTC, we deleted these eight residues. In this construct (d25-32), the 24 amino-terminal residues of the OTC leader peptide were joined to the amino terminus of mature OTC; arginine 23 (normally at position -2 relative to the MPP cleavage site and -10 relative to the MIP cleavage site) was now at position -2 from the MIP cleavage site (Fig. 4 a). Translation mixtures containing wild-type pOTC or d25-32 were incubated with the peptidases. Fig. 4 a shows that wild-type pOTC was processed in two steps by MPP and MIP (lanes 1-4). In contrast, d25-32 was not processed by either peptidase (lanes 5-8).

The Two Amino-Terminal Residues of the Octapeptide Are Sufficient to Restore Cleavage by MPP

To determine which residues in the OTC octapeptide are necessary to restore one- or two-step processing of pOTC, we deleted six (d27-32), four (d29-32), and two (d31-32) residues, serially, from the carboxyl terminus of the leader peptide. In these constructs, the 26, 28, and 30 amino-terminal residues of the pOTC leader peptide were joined to the amino terminus of mature OTC, so that arginine 23 was always at position -2 from the MPP cleavage site, and at positions -4, -6, and -8, respectively, from the MIP cleavage site (Fig. 4 a). Each of these deleted precursors was processed by MPP to intermediate species of slightly different sizes (lanes 10, 14, and 18, respectively), whose electrophoretic mobilities suggested that cleavage had occurred in all cases at the normal MPP site. Incubation of the d27-32 and d29-32 precursors with MPP plus MIP did not have any additional effect (lanes 12 and 16, respectively). In the case of the d31-32 precursor, however, we observed that most of the intermediate formed by MPP was converted by MIP to a size similar to that of mature OTC (lane 20). We determined the sites of MPP cleavage in d27-32 (Fig. 4 b) and of MIP cleavage in d31-32 (Fig. 4 c). d27-32 and d31-32 were synthesized in a reticulocyte lysate in the presence of [³H]-leucine and incubated with MPP (d27-32), or MPP plus MIP (d31-32). Upon SDS/PAGE and electroblotting, the bands

corresponding to iOTC (d27-32) and mature OTC (d31-32) were excised and sequenced directly. In the case of d27-32 (Fig. 4 b), release of [³H]leucine in cycles 7, 12, 13, and 15 indicated that MPP had cleaved this precursor at the normal iOTC amino terminus, i.e., between asn 24 and phe 25 (Sztul et al., 1987). In the case of d31-32 (Fig. 4 c), release of [³H]leucine in cycles 3, 8, 9, and 11 indicated that MIP had cleaved this protein between lys 32 and val 33, i.e., two residues downstream from the normal mature amino terminus, but precisely eight residues from the MPP cleavage site. Note that in the d31-32 precursor, the proline residue normally found at position 30 in the wild-type pOTC was changed to glutamine to avoid the possibility that this proline could interfere with cleavage at the mature amino terminus.

Only a Specific Octapeptide Can Direct Two-Step Processing of pOTC

To determine if an eight-amino acid "joint" between the MPP and MIP sites, independent of its primary amino acid sequence, is sufficient to support two cleavages, we substituted an octapeptide with four repeats of leucine-glutamine for the authentic pOTC octapeptide. These two amino acids were chosen because they are frequently found in intermediate octapeptides. The OTC precursor containing this artificial octapeptide [OTC-(art8)-OTC] was not cleaved by either peptidase (Fig. 5, lanes 5-8). When the MDH (residues 17-24) (OTC-(MDH8)-OTC) or the Fe/S (residues 25-32) (OTC-(Fe/S8)-OTC) octapeptide was substituted for the natural OTC octapeptide, however, two-step processing of these precursors occurred normally (lanes 9-12 and 13-16, respectively).

The pMUT Leader Peptide Is Not Processed when Joined to the Amino Terminus of Mature OTC

To define further the role played by the octapeptide and the amino terminus of the mature protein in directing one- or two-step processing, we constructed a chimera between a once-cleaved and a twice-cleaved protein. The 32 amino acid pMUT leader peptide (normally cleaved in one step by MPP) was joined to the amino terminus of mature OTC; arginine 31, found at position -2 relative to the mature MUT amino terminus, remained at -2 relative to the amino terminus of mature OTC (Fig. 6). This chimeric precursor was not processed by either peptidase (5-8).

The Mature Amino Terminus of MUT Can Restore Cleavage of MUT-OTC by MPP and MIP

A significant homology exists between the eight carboxy-terminal residues of the pOTC leader peptide (i.e., the octapeptide) and the eight amino-terminal residues of mature MUT (see Fig. 1): a hydrophobic residue is found at position 1 (phenylalanine in OTC; leucine in MUT); a positively charged residue is at position 2 (arginine in OTC; histidine in MUT); and the residues at positions 5, 6, and 7 are identical (glutamine, proline, and leucine, respectively). We also observed that some of the sequence features characteristics of the intermediate octapeptides are found within the eight amino-terminal residues of other mitochondrial proteins normally matured in one step (Hendrick et al., 1989). This suggested that the mature amino terminus of a once-cleaved protein might be a functional homologue of octapeptides. There-

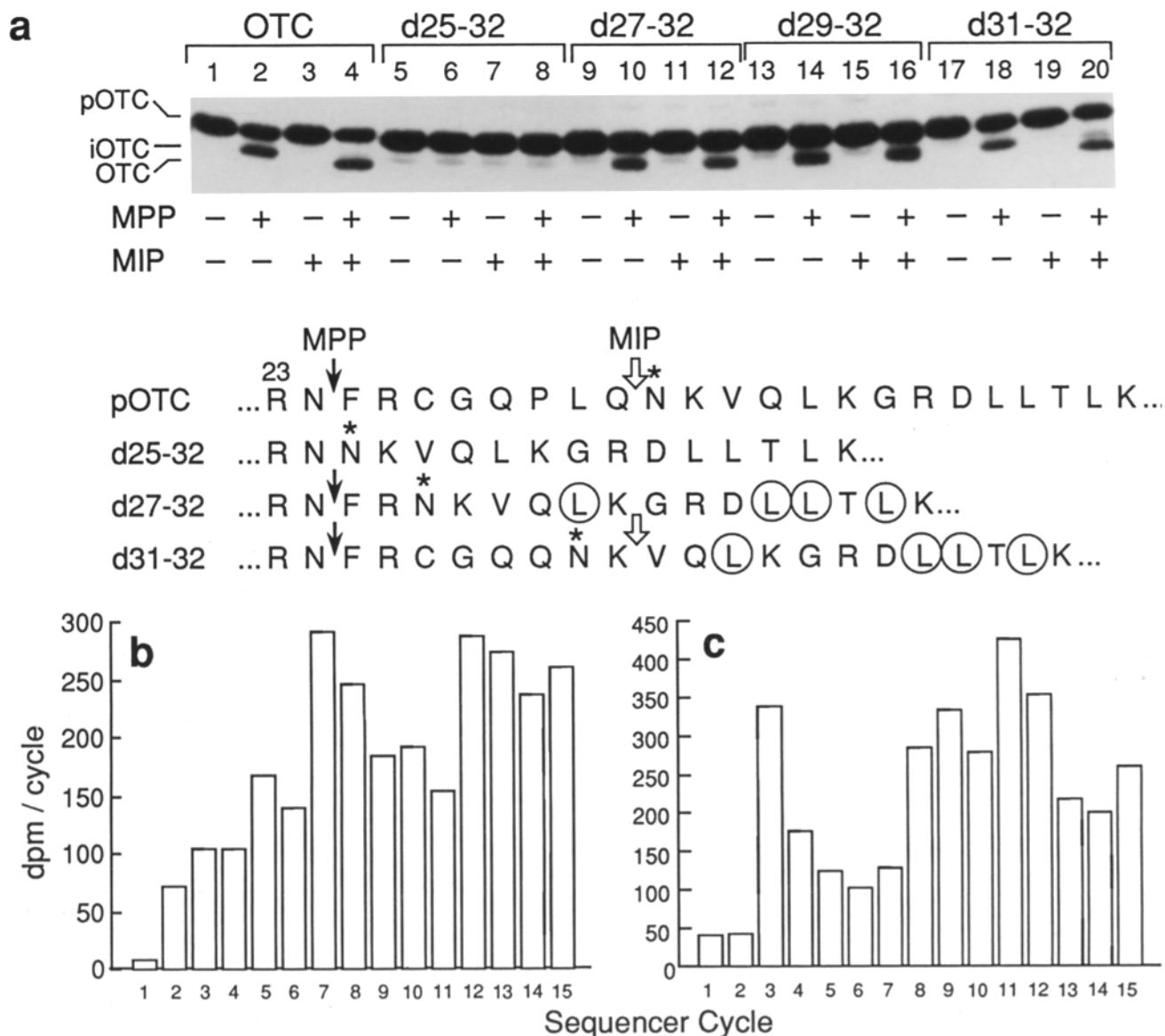


Figure 4. Deletion of the octapeptide blocks processing of pOTC. (a) The wild-type and deleted OTC precursors were incubated with MPP, MIP, and MPP plus MIP as indicated for 1 h at 27°C as described in Materials and Methods. The products of the processing reactions were analyzed directly by SDS/PAGE. The product of aberrant translation initiation at methionine 21 of the pOTC leader peptide is observed in lanes 5–8, 9, 11, 13, and 15, and has different positions depending on the length of the pOTC molecules.

(b and c) The d27-32 and d31-32 precursors were synthesized in the presence of [³H]leucine and incubated with MPP (d27-32) or MPP plus MIP (d31-32). The intermediate species formed upon processing of d27-32 (b) and the mature species formed upon processing of d31-32 (c) were subjected to radiosequencing, as described in Materials and Methods. The amino acid sequences of pOTC, d25-32, d27-32, and d31-32 are shown and labeled leucines are indicated by circled residues. The sites of MPP (solid arrow) and MIP (open arrow) cleavage are indicated. The asterisk marks the normal mature OTC amino terminus, and 23 marks arginine 23 in the pOTC leader peptide. Note that in d31-32, a glutamine has been substituted for proline as position 30.

fore, we constructed three chimeric precursors containing the pMUT leader peptide and, respectively, the two (MUT2-OTC), six (MUT6-OTC), and eight (MUT8-OTC) amino-terminal residues of mature MUT, joined to the amino terminus of mature OTC. In these constructs, arginine 31 was always at position –2 from the normal MPP cleavage site, and at positions –4, –8, and –10, respectively, from the mature OTC amino terminus (Fig. 6). Each of the three artificial precursors was processed by MPP to an intermediate species of slightly different size (lanes 10, 14, and 18, respec-

tively). Radiosequence analysis of the MUT2-OTC intermediate species (lane 10) indicated that MPP cleaved at the normal mature MUT amino-terminus, i.e., between leu 32 and leu 33 (data not shown). Incubation of the MUT2-OTC and MUT6-OTC precursors with MPP and MIP did not have any additional effect (lanes 12 and 16, respectively). Incubation of the MUT8-OTC precursor with both peptidases, however, led to conversion of the intermediate to mature OTC (lane 20). Thus, the two amino-terminal residues of mature MUT were sufficient to restore cleavage by MPP, whereas eight

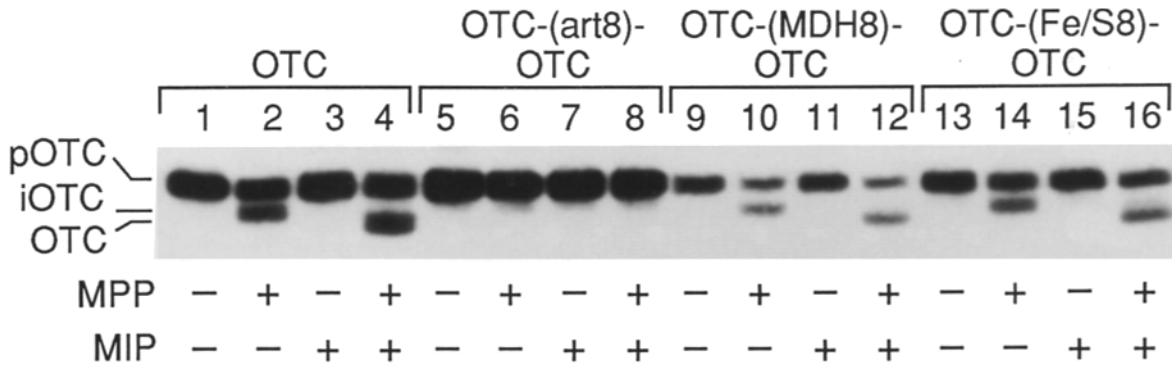


Figure 5. Only a specific octapeptide can direct two-step processing of pOTC. Experimental conditions are as in the legend for Fig. 4. The amino acid sequences of wild-type and chimeric OTC precursors are shown and the sites of MPP and MIP cleavage are indicated by arrows, as before. The asterisk marks the normal mature OTC amino terminus and 23 the arginine residue at position 23 in the pOTC leader peptide.

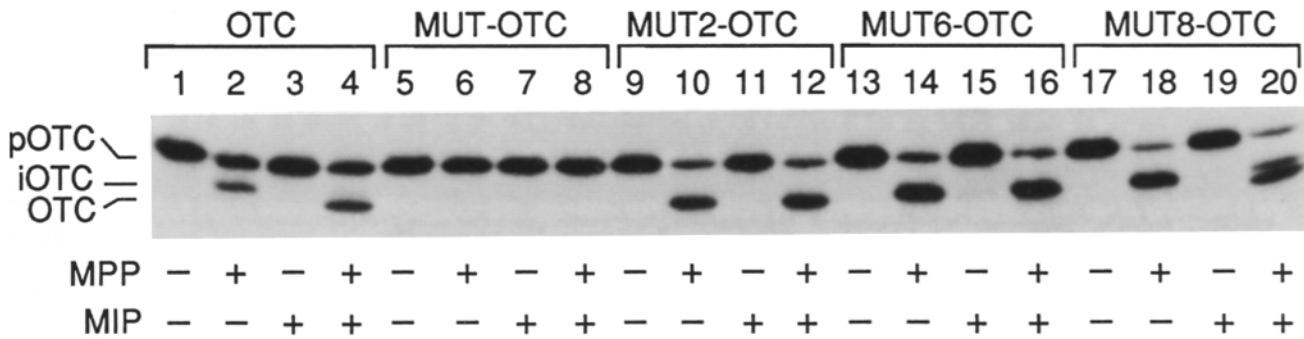


Figure 6. The pMUT leader peptide is not cleaved when joined to mature OTC. Experimental conditions are as in the legend for Fig. 4. The amino acid sequences of wild-type and chimeric OTC precursors are shown and the sites of MPP and MIP cleavage are indicated by arrows, as before. The site of MPP cleavage in MUT2-OTC was verified by radiosequence analysis (not shown). The asterisk marks the normal mature OTC amino terminus, 23 and 31 mark arginine 23 and arginine 31 in the pOTC and pMUT leader peptides, respectively.

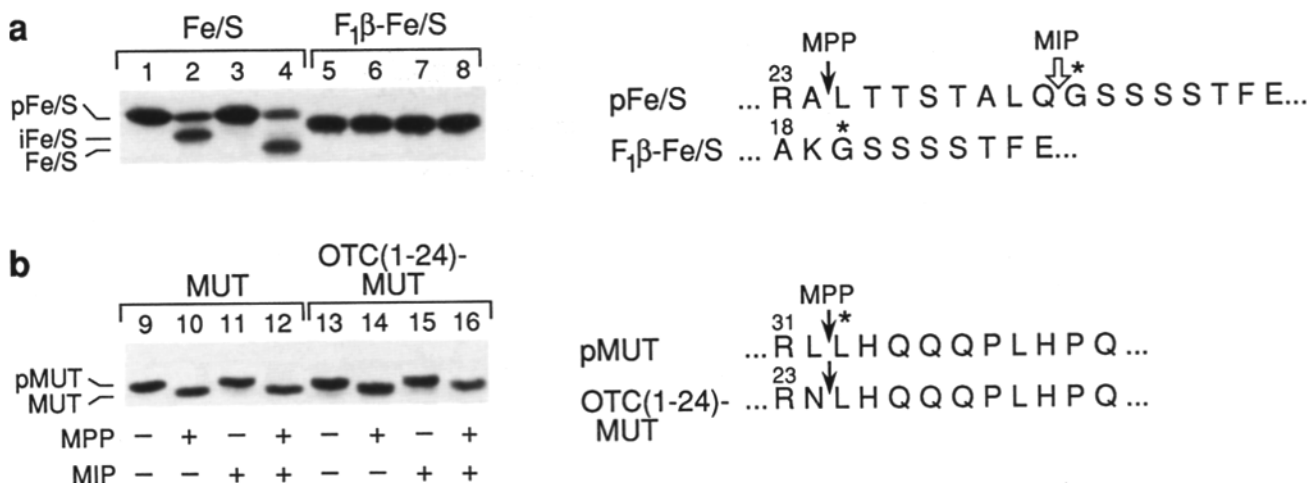


Figure 7. (a) The pF₁β leader peptide is not cleaved when joined to mature Fe/S. (b) The pOTC leader peptide is cleaved by MPP when joined to mature MUT. Experimental conditions are as in the legend for Fig. 4. The amino acid sequences of wild-type and chimeric precursors are shown. The asterisk marks the normal amino terminus of mature Fe/S and of mature MUT, respectively; 23 marks arginine 23 in the pOTC leader peptide; 31 marks arginine 31 in the pMUT leader peptide; 18 marks alanine 18 in the pF₁β leader peptide. The sites of MPP and MIP cleavage are indicated by the arrows, as before.

amino-terminal MUT residues could direct subsequent cleavage by MIP.

The pF₁β Leader Peptide Is Not Processed When Joined to the Amino Terminus of Mature Fe/S

To exclude the possibility that these observations pertained only to pOTC and pMUT, we also constructed a chimeric precursor bearing the leader peptide of yeast pF₁β, normally cleaved in one step (Vassarotti et al., 1987; Hendrick et al., 1989) (Fig. 3, lanes 5–8), joined to the mature portion

of *Neurospora* pFe/S, normally cleaved in two sequential steps (Hartl et al., 1986; Hendrick et al., 1989) (Fig. 7 a, lanes 1–4). As predicted from the d25-32 and MUT-OTC experiments above, the F₁β-Fe/S chimera was not cleaved by either peptidase (Fig. 7 a, lanes 5–8).

The pOTC Leader Peptide Is Cleaved by MPP When Joined to the Mature MUT Amino Terminus

Lack of cleavage of the d25-32 OTC, MUT-OTC, and F₁β-Fe/S precursors by MPP suggested that the mature amino

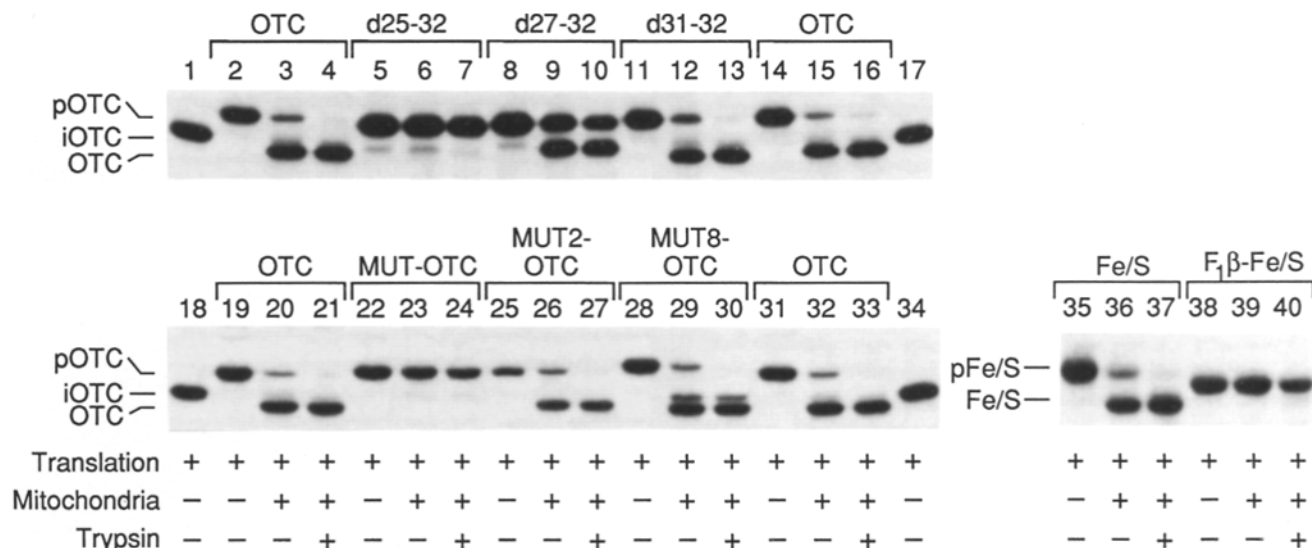


Figure 8. Processing of the mutant and chimeric precursors by isolated rat liver mitochondria. 6 μl of [³⁵S]methionine-labeled translation mixture was incubated with 4 μl of a suspension of freshly isolated rat liver mitochondria (20 mg/ml) for 20 min at 27° C as described in Materials and Methods (lanes 3, 6, 9, 12, 15, 20, 23, 26, 29, 32, 36, and 39). After import, aliquots were treated with trypsin (final concentration 40 μg/ml) for 10 min on ice and then supplemented with soybean trypsin inhibitor at a final concentration of 100 μg/ml (lanes 4, 7, 10, 13, 16, 21, 24, 27, 30, 33, 37, and 40). Products of the import reactions were analyzed directly by SDS/PAGE and fluorography. In lanes 1, 17, 18, and 34, Met-iOTC was used as a standard for the position of normal iOTC. Amounts of pOTC equivalent to those added to the import reactions were fully digested by adding 40 μg/ml trypsin to the reticulocyte lysate for 10 min at 0° C (not shown). When Triton X-100 was added to solubilize the mitochondria before trypsin treatment, iOTC was totally degraded, whereas 40–50% of mature OTC was resistant (not shown).

terminus of a twice-cleaved precursor is structurally incompatible with cleavage by MPP. The structural requirements that restore MPP cleavage of these constructs are provided by the two amino-terminal residues of the octapeptide or of mature MUT. It follows that the 24 amino-terminal residues of pOTC (not including the octapeptide) should be functionally homologous to the entire pMUT leader peptide and should be cleaved by MPP when joined to mature MUT. In this construct, arginine 23 in the pOTC leader peptide is placed at position -2 from the amino terminus of mature MUT (Fig. 7 b). The wild-type MUT (lane 9) and the OTC(1-24)-MUT (lane 13) precursors were incubated with the purified peptidases. Both precursors were efficiently converted to mature MUT upon incubation with MPP (lanes 10 and 14, respectively). Incubation of precursors with both peptidases (lanes 12 and 16) did not have any additional effect.

Processing of Mutant Precursors by Isolated Rat Liver Mitochondria Is Consistent with Processing by Purified Peptidases

Several studies have shown that precursor proteins are in native, folded conformations after translation in a reticulocyte lysate (Skerjanc et al., 1990). Therefore, lack of processing of d25-32, MUT-OTC, and $F_1\beta$ -Fe/S precursors in the soluble system might have occurred because these proteins fold in such a way that leaders become inaccessible to cleavage by MPP. On the other hand, it has been shown that precursors are unfolded during translocation into intact mitochondria (Hartl et al., 1989) and that refolding and assembly generally follow proteolytic processing (Ostermann et al., 1989). Thus, we asked whether those mutant precursors that were not cleaved in the soluble system could undergo proteolytic processing by intact mitochondria. Fig. 8 shows that the d25-32, MUT-OTC, and $F_1\beta$ -Fe/S precursors were imported by isolated mitochondria, as indicated by protection of significant amounts of these three precursors from externally added trypsin (lanes 7, 24, and 40). Nevertheless, no production of mature OTC or mature Fe/S was observed in intact mitochondria, identical to the results with the purified peptidases and consistent with the idea that the mature OTC and Fe/S amino termini are incompatible with the usual site of cleavage of MPP.

We further asked whether the patterns of processing observed in the soluble system with other precursors were also consistent with processing by intact mitochondria. The d27-32 (lanes 8-10) and MUT2-OTC (lanes 25-27) were cleaved to intermediate-sized species, apparently identical to those obtained upon incubation with MPP or with MPP plus MIP (see Fig. 4, lanes 10 and 12 and Fig. 6, lanes 10 and 12). The d31-32 (lanes 11-13) and MUT8-OTC (lanes 28-30) precursors were processed to intermediate- and mature-sized species, as observed upon incubation with MPP plus MIP (see Fig. 4, lane 20 and Fig. 6, lane 20).

Discussion

A number of mitochondrial precursors have two-domain leader peptides (von Heijne et al., 1989; Hendrick et al., 1989) that are cleaved in subsequent steps by two independent matrix peptidases (MPP and MIP) (Kalousek et al.,

1988), whereas the majority of precursors are cleaved in one step by MPP alone (Hawlitsek et al., 1988; Yang et al., 1988; Ou et al., 1989). While two-step processing by MPP and an intermembrane space peptidase was shown to direct the intramitochondrial sorting of some of the precursors destined for the intermembrane space (Hartl et al., 1986), no functional explanation was available before this study for two-step processing by the two known matrix peptidases of precursors destined for other mitochondrial subcompartments. That a higher order protein structure might be responsible for recognition and cleavage of mitochondrial leader peptides by the matrix peptidases has been postulated for some time (von Heijne et al., 1989; Hendrick et al., 1989). Consistent with this idea, for example, was the observation that deletions distal to the mature amino terminus of the yeast F_1 ATPase β -subunit interfered with proteolytic cleavage (Vassarotti et al., 1987), even when as many as 17 normal residues were present downstream from the cleavage site.

We hypothesized that structural features in the mature proteins might determine whether one- or two-step processing of mitochondrial precursors occurs (Hendrick et al., 1989). A critical step in testing this hypothesis was the separation of the two processing activities found in the mitochondrial matrix. We characterized previously two partially purified fractions of rat liver mitochondrial matrix (Kalousek et al., 1988). The first of these fractions was shown to contain iOTC-forming activity (as well as iMDH-forming and mature β propionyl-CoA carboxylase-forming activity), i.e., MPP activity [previously called P_1 (Kalousek et al., 1988) or MPP-I (Kleiber et al., 1990)]. The second was shown to contain both MPP activity and mature OTC-forming activity (as well as mature MDH-forming activity), i.e., MIP activity (previously called P_2 [Kalousek et al., 1988] or MPP-II [Kleiber et al., 1990]).

We have now separated these two activities and have purified MPP to homogeneity. Our final MPP preparation contains two polypeptides of 55 and 52 kD (Fig. 2, lane 7). Rat liver MPP has been reported previously to consist of a heterodimeric complex containing two subunits of 55 and 52 kD (Ou et al., 1989). In *Neurospora* and yeast, two proteins are also required for processing activity, MPP, the catalytic component, and PEP, the processing enhancing protein (Hawlitsek et al., 1988; Pollock et al., 1988; Yang et al., 1988). Amino acid sequence analysis of tryptic peptides obtained from the 55-kD subunit of our MPP preparation allowed us to isolate a cDNA clone whose deduced amino acid sequence bears 36% identity with yeast and *Neurospora* MPP, suggesting that the 55-kD subunit is the catalytic component of rat mitochondrial processing peptidase (Kleiber et al., 1990). The 52-kD protein might be a stimulator of the processing activity, similar to PEP in *Neurospora*.

Further studies will be required to establish the equivalence of the 55- and 52-kD subunits of the rat enzyme to MPP and PEP of yeast and *Neurospora*. Nevertheless, these data, together with the observation that the processing activity of a crude matrix fraction can be inhibited with antibodies raised against a partially purified MPP fraction (data not shown) and that correct cleavage of several precursors is catalyzed by purified MPP (see Fig. 3), indicate that we have purified the bona fide rat liver mitochondrial processing peptidase.

We have also prepared a fraction enriched in MIP activity and, importantly, free of detectable MPP activity. Because, in the case of twice-cleaved precursors, formation of the intermediate is required for formation of the mature protein (Sztul et al., 1987), it was not surprising that the MIP fraction did not display any proteolytic activity upon incubation with pOTC, unless MPP was also added. Under the latter condition, mature OTC was formed by quantitative conversion of iOTC.

These data may raise the question whether MIP is a cofactor required by MPP to complete maturation of twice-cleaved precursors. This seems most unlikely because the MIP fraction was capable of processing Met-iOTC to mature OTC in the absence of MPP, indicating that MIP is an independent peptidase and that the mitochondrial intermediate proteins are the substrate for this enzyme.

The second facet of our study was to use the two peptidase fractions to analyze the patterns of processing of artificial precursors specifically designed to emphasize the structural requirements for two-step processing (a summary of cleavage of these precursors by MPP and MIP is shown in Fig. 9). We have asked whether the intermediate octapeptides, found at the carboxy terminus of the leader peptides of twice-cleaved precursors, are necessary for formation of the corresponding mature proteins. Alternatively, we have tested whether the placement of an arginine residue at -2 from the mature amino terminus of a twice-cleaved precursor might be sufficient to direct cleavage of the leader peptide by MPP alone, as occurs in the majority of once-cleaved precursors.

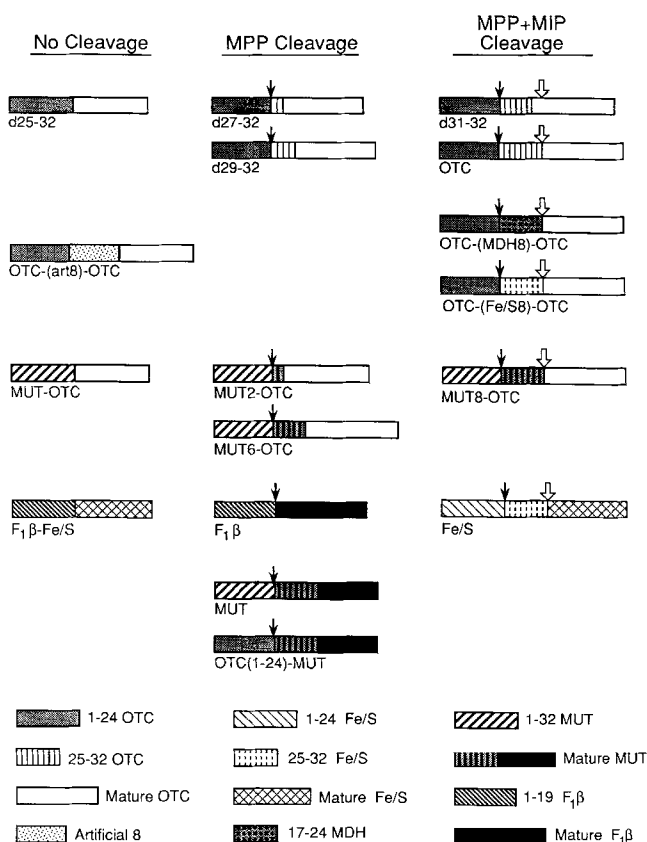


Figure 9. Summary of cleavage of wild-type, deleted, and chimeric precursors by MPP and MIP.

In answer to these questions, we observed a total lack of processing when the pOTC octapeptide was deleted (d25-32) or when the entire pMUT leader peptide was joined to mature OTC (MUT-OTC), even though an arginine residue is present in these constructs at position -2 from the mature amino terminus, such that the general characteristics of the MPP cleavage site are maintained. Because a required primary amino acid sequence has not been identified on the carboxy-terminal side of MPP cleavage sites and because the amino acids found at the mature amino termini of twice-cleaved proteins are not significantly different from those found at the amino terminus of once-cleaved proteins (von Heijne et al., 1989; Hendrick et al., 1989), we conclude that a higher order protein structure at the amino terminus of mature OTC is incompatible with cleavage by MPP. Moreover, lack of cleavage of the $F_1\beta$ -Fe/S chimera strongly indicates that a similar structural constraint exists at the amino terminus of mature Fe/S. Therefore, the octapeptides normally found in these precursors must function to provide a structure compatible with MPP cleavage.

Cleavage of d25-32 and MUT-OTC precursors by MPP was restored by re-inserting the two amino-terminal residues of the OTC octapeptide (phenylalanine and arginine) or of mature MUT (leucine and histidine) between the MPP cleavage site and the mature OTC amino terminus (see the d27-32 and MUT2-OTC experiments above). Although these data are consistent with the idea that these pairs of residues are needed simply to complete the MPP cleavage site, this seems unlikely because there is no general requirement for phenylalanine-arginine (or leucine-histidine) on the carboxy-terminal side of MPP cleavage sites (von Heijne et al., 1989; Hendrick et al., 1989). On the other hand, a hydrophobic residue (generally phenylalanine) is always found at the amino terminus of the intermediate octapeptides (Hendrick et al., 1989). Thus, an alternative explanation is that, in the d27-32 precursor, the two amino-terminal residues of the octapeptide, joined to the six amino-terminal residues of mature OTC, reconstitute a partially functional octapeptide that can, in fact, restore cleavage by MPP at the normal iOTC amino terminus. Moreover, in the case of the d31-32 precursor, a fully functional (i.e., cleavable) octapeptide is produced by joining the six amino-terminal residues of the OTC octapeptide to the two amino-terminal residues of mature OTC. In the same way, the two and eight amino-terminal residues of mature MUT, sharing a significant homology with the corresponding residues in the OTC octapeptide, can reconstitute a partially or fully functional, respectively, octapeptide when joined to the amino terminus of mature OTC. Finally, the fact that pOTC containing an artificial octapeptide made of four repeats of leucine and glutamine is not processed by MPP, while pOTC containing the MDH or the Fe/S octapeptide is processed normally, further indicates that structural features of the octapeptide, not simply specific amino acids immediately carboxy-terminal to the MPP cleavage site, are responsible for directing proteolytic processing.

Thus, our data indicate that a specific octapeptide downstream from the MPP cleavage site is required for cleavage of twice-cleaved proteins by MPP. In contrast, when the 24 amino-terminal residues of the pOTC leader peptide (not including the octapeptide) are joined directly to the amino terminus of mature MUT (a once-cleaved protein), cleavage by MPP occurs normally. Therefore, we can extend our conclu-

sion to say that a specific structure defined either by the mature amino terminus of once-cleaved proteins or by the octapeptide of twice-cleaved precursors, determines recognition and cleavage by MPP.

We do not know the nature of the structure in the mature portions of OTC and Fe/S (and probably in other mitochondrial proteins matured in two steps) that is responsible for requiring a cleavable octapeptide in these precursor molecules. On the other hand, because this juxtaposition of cleavable octapeptide to mature amino terminus has been conserved during evolution of mitochondrial proteins and because it has led to the development of an enzymatic system (i.e., MIP) devoted exclusively to its activation, we believe it must play a crucial role in determining the fate and function of the mature protein.

We thank the Yale School of Medicine Protein and Nucleic Acid Chemistry Facility for synthesis of oligonucleotide primers and for amino acid sequencing, and Connie Woznick for expert secretarial assistance.

This work was supported by a grant from the National Institutes of Health (DK09527).

Received for publication 4 April 1990 and in revised form 29 October 1990.

References

- Chu, T. W., P. M. Grant, and A. W. Strauss. 1987. The role of arginine residues in the rat mitochondrial malate dehydrogenase transit peptide. *J. Biol. Chem.* 262:12806-12811.
- Conboy, J. G., and L. E. Rosenberg. 1981. Posttranslational uptake and processing of *in vitro* synthesized ornithine transcarbamylase precursor by isolated rat liver mitochondria. *Proc. Natl. Acad. Sci. USA.* 78:3073-3077.
- Conboy, J. G., W. A. Fenton, and L. E. Rosenberg. 1982. Processing of pre-ornithine transcarbamylase requires a zinc-dependent protease localized to the mitochondrial matrix. *Biochem. Biophys. Res. Commun.* 105:1-7.
- Fenton, W. A., A. M. Hack, D. Helfgott, and L. E. Rosenberg. 1984. Biogenesis of the mitochondrial enzyme methylmalonyl-CoA mutase. Synthesis and processing of a precursor in a cell-free system and in cultured cells. *J. Biol. Chem.* 259:6616-6621.
- Grant, P. M., J. Tellam, V. L. May, and A. W. Strauss. 1986. Isolation and nucleotide sequence of a cDNA clone encoding rat mitochondrial malate dehydrogenase. *Nucleic Acids Res.* 14:6053-6066.
- Harnish, U., H. Weiss, and W. Sebald. 1985. The primary structure of the iron-sulfur subunit of ubiquinol-cytochrome c reductase from *Neurospora*, determined by cDNA and gene sequencing. *Eur. J. Biochem.* 149:95-99.
- Hartl, F. -U., B. Schmidt, E. Wachter, H. Weiss, and W. Neupert. 1986. Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. *Cell.* 47:939-951.
- Hartl, F. -U., J. Ostermann, B. Guiard, and W. Neupert. 1987. Successive translocation in and out of the mitochondrial matrix: targeting of proteins to the intermembrane space by a bipartite signal peptide. *Cell.* 51:1027-1037.
- Hawlitcshek, G., H. Schneider, B. Schmidt, M. Tropschug, F. -U. Hartl, and W. Neupert. 1988. Mitochondrial protein import: Identification of processing peptidase and of PEP, a processing enhancing protein. *Cell.* 53:785-806.
- Hendrick, J. P., P. E. Hodges, and L. E. Rosenberg. 1989. Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: Leader peptides cleaved by two matrix proteases share a three-amino acid motif. *Proc. Natl. Acad. Sci. USA.* 86:4056-4060.
- Horwich, A. L., W. A. Fenton, K. R. Williams, F. Kalousek, J. P. Kraus, R. F. Doolittle, W. Konigsberg, and L. E. Rosenberg. 1984. Structure and expression of a complementary DNA for the nuclear coded precursor of human mitochondrial ornithine transcarbamylase. *Science (Wash. DC).* 224:1068-1074.
- Jansen, R., F. Kalousek, W. A. Fenton, L. E. Rosenberg, and F. D. Ledley. 1989. Cloning of a full-length methylmalonyl-CoA mutase from a cDNA library using the polymerase chain reaction. *Genomics.* 4:198-205.
- Jensen, R. E., and M. P. Yaffe. 1988. Import of proteins into yeast mitochondria: the nuclear MAS2 gene encodes a component of the processing protease that is homologous to the MAS1-encoded subunit. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3863-3871.
- Kalousek, F., J. P. Hendrick, and L. E. Rosenberg. 1988. Two mitochondrial matrix proteases act sequentially in the processing of mammalian matrix enzymes. *Proc. Natl. Acad. Sci. USA.* 85:7536-7540.
- Kleiber, J., F. Kalousek, M. Swaroop, and L. E. Rosenberg. 1990. The general mitochondrial matrix processing protease from rat liver: structural characterization of the catalytic subunit. *Proc. Natl. Acad. Sci. USA.* 87:7978-7982.
- Kozak, M. 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol.* 196:947-950.
- Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* 262:10035-10038.
- Mullis, K. B., and F. A. Faloona. 1989. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. In *Recombinant DNA Methodology*, R. Wu, L. Grossman, and K. Moldave, editors. Academic Press, Inc., New York. 189-204.
- Ostermann, J., A. L. Horwich, W. Neupert, and F. -U. Hartl. 1989. Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis. *Nature (Lond.).* 341:125-130.
- Ou, W. -J., A. Ito, H. Okazaki, and T. Omura. 1989. Purification and characterization of a processing protease from rat liver mitochondria. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2605-2612.
- Pfanner, N., and W. Neupert. 1990. The mitochondrial protein import apparatus. *Annu. Rev. Biochem.* 59:331-353.
- Pollock, R. A., F. -U. Hartl, M. -Y. Cheng, J. Ostermann, A. Horwich, and W. Neupert. 1988. The processing peptidase of yeast mitochondria: the two cooperating components MPP and PEP are structurally related. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3493-3500.
- Rosenberg, L. E., W. A. Fenton, A. L. Horwich, F. Kalousek, and J. P. Kraus. 1987. Targeting of nuclear-encoded proteins to the mitochondrial matrix: Implications for human genetic defects. *Ann. NY Acad. Sci.* 488:99-108.
- Schneider, H., M. Arretz, E. Wachter, and W. Neupert. 1990. Matrix processing peptidase of mitochondria. Structure-function relationships. *J. Biol. Chem.* 265:9881-9887.
- Skerjanc, I. S., W. P. Sheffield, S. K. Randall, J. R. Silvius, and G. Shore. 1990. Import of precursor proteins into mitochondria: site of polypeptide unfolding. *J. Biol. Chem.* 265:9444-9451.
- Sztl, E. S., J. P. Hendrick, J. P. Kraus, D. Wall, F. Kalousek, and L. E. Rosenberg. 1987. Import of rat ornithine transcarbamylase precursor into mitochondria: two-step processing of the leader peptide. *J. Cell Biol.* 105:2631-2639.
- Sztl, E. S., Chu, T. W., Strauss, A. W., and Rosenberg, L. E. 1988. Import of the malate dehydrogenase precursor by mitochondria. Cleavage within leader peptide by matrix protease leads to formation of intermediate-sized form. *J. Biol. Chem.* 263:12085-12091.
- Takeda, M., A. Vassarotti, and M. G. Douglas. 1985. Nuclear genes coding the yeast mitochondrial adenosine triphosphatase complex. Primary sequence analysis of ATP2 encoding the F₁-ATPase β -subunit precursor. *J. Biol. Chem.* 260:15458-15465.
- Takeda, M., A. Vassarotti, and M. G. Douglas. 1986. Nuclear genes coding the yeast mitochondrial adenosine triphosphatase complex. Primary sequence analysis of ATP2 encoding the F₁-ATPase β -subunit precursor. *J. Biol. Chem.* 261:10466.
- Tropschug, M., D. W. Nicholson, F. -U., Hartl, H. Kohler, N. Pfanner, E. Wachter, and W. Neupert. 1988. Cyclosporin A-binding protein (Cylophilin) of *Neurospora crassa*. One gene codes for both the cytosolic and mitochondrial forms. *J. Biol. Chem.* 263:14433-14440.
- Verner, K., and G. Schatz. 1988. Protein translocation across membranes. *Science (Wash. DC).* 241:1307-1313.
- Vassarotti, A., W. -J. Chen, C. Smaglia, and M. G. Douglas. 1987. Sequences distal to the mitochondrial targeting sequences are necessary for the maturation of the F₁-ATPase β -subunit precursor in mitochondria. *J. Biol. Chem.* 262:411-418.
- von Heijne, G., J. Steppuhn, and R. G. Herrmann. 1989. Domain structure of mitochondrial and chloroplast targeting peptides. *Eur. J. Biochem.* 180:535-545.
- Witte, C., R. E. Jensen, M. P. Jaffe, and G. Schatz. 1988. MAS1, a gene essential for yeast mitochondrial assembly, encodes a subunit of the mitochondrial processing protease. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1439-1447.
- Yang, M., R. E. Jensen, M. P. Yaffe, W. Opplinger, and G. Schatz. 1988. Import of proteins into yeast mitochondria: the purified matrix processing protease contains two subunits which are encoded by the nuclear MAS1 and MAS2 genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3857-3862.