Amino-terminal Octapeptides Function as Recognition Signals for the Mitochondrial Intermediate Peptidase*

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The vast majority of mitochondrial proteins are encoded in the nucleus and synthesized in the cytoplasm as larger precursors bearing amino-terminal leader peptides that contain the information necessary for mitochondrial localization (Rossenbarg et al., 1987; Verner and Schatz, 1988; Pfannen and Neupert, 1990). Upon import to the mitochondrial matrix, most leader peptides are proteolytically cleaved in one step by the mitochondrial processing peptidase (MPP)1 (Hawitschek et al., 1988; Yang et al., 1988; Pollock et al., 1988; Ou et al., 1989). A number of precursors are cleaved in sequential steps by two independent peptidases (Hurt et al., 1986; Hartl et al., 1986; Sztul et al., 1987, 1988; Tropschug et al., 1988; Fu et al., 1990). These precursors are initially processed by MPP to intermediate-sized forms; the intermediates are sequentially cleaved by the mitochondrial intermediate peptidase (MIP) leading to formation of mature proteins (Kalousek et al., 1988; Isaya et al., 1991).

Although the functional correlation among twice-cleaved proteins is not understood, these precursors are characterized by the presence of specific octapeptides between the MPP and the MIP cleavage sites (Hendrick et al., 1989; von Heijne et al., 1989; Gavel and von Heijne, 1990). The octapeptides always contain a hydrophobic residue, generally phenylalanine, at position 1, and a small residue, serine, threonine, or glycine, at position 4 (corresponding to positions −8 and −5, respectively, relative to the amino terminus of the mature protein generated from the intermediate molecule).

We have shown previously that cleavage of a number of precursors by the mitochondrial processing peptidase (MPP) requires an intermediate octapeptide (FXXSXXXX) between the MPP cleavage site and the mature protein amino terminus. We show now that these octapeptides, present at the amino termini of the intermediates, directly recognize those substrates for the mitochondrial intermediate peptidase (MIP), leading to formation of mature proteins. Synthetic peptides, corresponding to the intermediate octapeptides of human ornithine transcarbamylase (OTC) and of Neurospora cytochrome c reductase Fe/S subunit (Fe/S), inhibit the processing activity of purified rat liver MIP in vitro, without affecting MPP activity; this indicates that the octapeptides can be recognized by MIP independent of the presence of the corresponding mature peptides and interact with a site that is crucial for MIP activity. MIP activity is not inhibited by a peptide lacking the amino-terminal hydrophobic residue, while substitution of such a residue by a polar amino acid causes a 10-fold reduction in the efficiency of MIP inhibition. To analyze the requirements for removal of the octapeptide from the intermediate protein by MIP, artificial intermediates were synthesized and subjected to in vitro processing by purified MIP. The octapeptide can be cleaved by MIP only when the amino-terminal hydrophobic residue is also the amino terminus of the intermediate. Further, when the OTC octapeptide is joined to the mature amino terminus of another twice-cleaved precursor (pFe/S; rat malate dehydrogenase, pMDH), the chimeric intermediate is cleaved by MIP to the corresponding mature-sized protein. When the OTC octapeptide is joined to the mature amino terminus of a once-cleaved precursor (yeast F1-β-ATPase, pF1-β), however, this intermediate is not cleaved by MIP; rather, it is processed by MPP to mature-sized F1-β. Therefore, amino-terminal octapeptides can be cleaved by MIP only within the structural context of twice-cleaved precursors.

The vast majority of mitochondrial proteins are encoded in the nucleus and synthesized in the cytoplasm as larger precursors bearing amino-terminal leader peptides that contain the information necessary for mitochondrial localization (Rossenbarg et al., 1987; Verner and Schatz, 1988; Pfannen and Neupert, 1990). Upon import to the mitochondrial matrix, most leader peptides are proteolytically cleaved in one step by the mitochondrial processing peptidase (MPP)1 (Hawitschek et al., 1988; Yang et al., 1988; Pollock et al., 1988; Ou et al., 1989). A number of precursors are cleaved in sequential steps by two independent peptidases (Hurt et al., 1986; Hartl et al., 1986; Sztul et al., 1987, 1988; Tropschug et al., 1988; Fu et al., 1990). These precursors are initially processed by MPP to intermediate-sized forms; the intermediates are sequentially cleaved by the mitochondrial intermediate peptidase (MIP) leading to formation of mature proteins (Kalousek et al., 1988; Isaya et al., 1991).

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We have shown previously that the mature amino terminus of a twice-cleaved precursor is structurally incompatible with cleavage by MPP and that the octapeptide functions in these precursors to provide a compatible MPP cleavage site (Isaya et al., 1991). Gavel and von Heijne (1990) have further shown that the mature amino terminus of a twice-cleaved precursor contains more positively and less negatively charged amino acids than the mature amino terminus of a once-cleaved precursor. These authors suggested that the octapeptides may be necessary to separate the MPP cleavage site from a positively charged structure in the mature protein which would interfere with recognition by MPP.

Further data indicate that the intermediate octapeptides are the functional homologues of the mature amino termini of once-cleaved precursors. Yang et al. (1991) have shown that purified yeast MPP correctly cleaves a chemically synthesized mitochondrial leader peptide when no more than eight amino acids are present downstream from the MPP cleavage site. We have shown that the eight amino-terminal residues of the mature amino terminus of a once-cleaved precursor can substitute for the octapeptide in a twice-cleaved precursor (Isaya et al., 1991).

On the other hand, after providing the structural requirements for cleavage of the precursor by MPP, the octapeptides

1 The abbreviations used are: MPP, mitochondrial processing peptidase; MIP, mitochondrial intermediate peptidase; OTC, ornithine transcarbamylase; pOTC, precursor for OTC; iOTC, intermediate OTC; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; pMDH, precursor for rat malate dehydrogenase.
are independently cleaved by MIP (Isaya et al., 1991). This suggests that the octapeptide may also play a role in directing maturation of intermediate proteins by MIP. We have pos-
tulated that the octapeptide functions as specific signal for MIP and that the presence of the hydrophobic residues at the amino terminus of the octapeptide is important for MIP recognition.

We have now analyzed whether synthetic octapeptides may function as inhibitors of MIP activity in vitro and whether the presence of amino-terminal octapeptides is necessary and sufficient for cleavage of passenger proteins by MIP. We show that the octapeptides are recognized by MIP independent of the presence of the corresponding mature proteins. However, structural elements in the amino terminus of these proteins are required for cleavage of intermediates by MIP.

EXPERIMENTAL PROCEDURES

Materials—Peptides were synthesized on an Applied Biosystems Solid-Phase Peptide Synthesizer. Purification was by reverse-phase high performance liquid chromatography, and the peptides were characterized by amino acid analysis and fast atom bombardment mass spectrometry. Random peptides A and B were purchased from Sigma. Oligonucleotides were synthesized with an Applied Biosystems DNA Synthesizer (model 380A). The reagents for in vitro transcription and translation were obtained as described (Isaya et al., 1991).

In Vitro Processing—Rat liver MPP and MIP were purified from mitochondrial matrix as described (Isaya et al., 1991). Plasmids for in vitro synthesis of mitochondrial precursors contained the cDNAs encoding the precursor for human ornithine transcarbamylase (pOTC) (Horwich et al., 1984), rat malate dehydrogenase (pMDH) (Chu et al., 1987), Neurospora crassa ubiquinol cytochrome c reductase (pFe/S) subunit (pFe/S) (Hartl et al., 1986), and Saccharomyces cereuisiae F, ATPase subunit b (pF, b) (Bedwell et al., 1989), respectively, joined to the SP6 promoter. The cDNAs were transcribed in vitro, and mRNAs were translated in rabbit reticulocyte lysates in the presence of [35S]methionine. The processing reactions contained 1 μl of translation product, 0.1 mM MnCl₂, and 0.1 mM ZnCl₂ in a final volume of 10 μl of 10 mM Hepes buffer, pH 7.4, at 4 °C. Peptides were resuspended in 10 mM Hepes buffer, pH 7.4, and added to the processing reactions at the required concentrations. MPP and/or MIP were added, and the reactions were incubated at 27 °C for 15 min. The products of the reactions were analyzed by SDS/PAGE, fluorography, and densitometry of fluorograms.

In Vitro Mutagenesis—Construction of Met-(OTC26-32)-OTC has been described before (Isaya et al., 1991). This intermediate protein is translated from an initiating methionine that substitutes for the phenylalanine normally present at the amino terminus of the OTC octapeptide. The methionine is at position -8 relative to the amino terminus of mature OTC. Met-(OTC26-32)-OTC is called ‘iOTC’ in Figs. 2, 3, and 5. Met-(OTC25-32)-OTC was constructed essentially in the same way, except in this case an initiating methionine was added in front of the phenylalanine at the amino terminus of the OTC octapeptide. This methionine is at position -9 relative to the mature OTC amino terminus.

Construction of intermediate proteins containing the Met-(OTC26-32) octapeptide joined to the mature amino terminus of pMDH (Met-(OTC26-32)-MDH), pFe/S (Met-(OTC26-32)-Fe/S), and pF, b (Met-(OTC26-32)-F, b), respectively, was performed via polymerase chain reaction amplification of modified DNA sequences. Forward primers were complementary to the DNA sequences encoding the amino termini of mature proteins, mature Fe/S, and mature F, b, respectively. A noncomplementary extension at the 5' end of each primer contained a convenient restriction site, a consen-
sus sequence (Kozak, 1987), an ATG codon, and the sequence encoding residues 2-8 of the octapeptide. Antisense primers contained a convenient restriction site in the sequence encoding mature MDH, mature Fe/S, and mature F, b, respectively. These DNA fragments were coamplified containing the sequences encoding the Met-(OTC26-32) octapeptide joined directly to the amino terminus of mature MDH, mature Fe/S, and mature F, b, respectively. Vectors containing the pMDH, pFe/S, and pF, b cDNAs under the control of the SP6 promoter were digested as required to delete the leader peptide coding sequences from the cDNAs and to provide the bulk of the coding sequence and plasmid backbone. Each amplified fragment was subcloned into the appropriate vector, and the mutational alterations were verified by sequencing. Constructs were transcribed and translated in vitro, and the radiolabeled intermediate proteins were subjected to in vitro processing by purified rat liver MPP and MIP as described (Kalousek et al., 1988, Isaya et al., 1991). In a typical experiment, 1 μl of translation mixture was incubated with purified MPP and/or MIP in a final volume of 10 μl of 10 mM Hepes, pH 7.4, in the presence of 0.1 mM MnCl₂ and 0.1 mM ZnCl₂ for 1 h at 27 °C. The products of the processing reactions were analyzed by SDS/PAGE and fluorography. To increase the electrophoretic resolution between precursor, intermediate, and mature forms of F, b, we also synthesized pF, β and Met-(OTC26-32)-F, β proteins in which the 245 carboxyl-terminal amino acids were deleted, corresponding to species of approximately the same size of pOTC and Met-iOTC, respectively. The full length pF, β and Met-(OTC26-32)-F, β cDNAs, under the control of the SP6 promoter, were polymerase chain reaction-amplified using SP6 primer and an antisense primer complementary to the sequence coding for residues 350-354 of mature F, β, with a noncomplementary extension at the 5' end containing a stop codon. After removal of excess SP6 primers by spin dialysis using a Centricon 30 apparatus, the polymerase chain reaction products were transcribed directly, and mRNA were translated as described above.

RESULTS

Synthetic Octapeptides Inhibit Processing by Purified Rat Liver MIP in Vitro—We initially tested one peptide corresponding to residues 25-34 of pOTC (OTC25-34), i.e. the octapeptide and the 2 amino-terminal residues of mature OTC, and a second peptide corresponding to residues 31-40 of pOTC (OTC31-40), i.e. the 2 carboxyl-terminal residues of the octapeptide and the 8 amino-terminal residues of mature OTC (see Fig. 1 for peptide sequences). Because these peptides had been designed initially to perform affinity chro-
matography of MIP, they also contained a cysteine at the carboxyl terminus, while the cysteine normally at position 27 in the pOTC sequence was changed to a glutamine in peptide OTC(25-34).

In vitro-translated pOTC was incubated in the presence of MPP and increasing concentrations of peptide OTC(25-34) or peptide OTC(31-40) (Fig. 1). In the absence of peptides, pOTC was normally processed to intermediate OTC (iOTC) by MPP. Formation of equivalent amounts of iOTC by MPP was obtained in the presence of a 50 μM or 100 μM concentration of either peptide.

In the absence of peptide, incubation of pOTC with MPP and MIP led to nearly quantitative conversion of iOTC to mature OTC by MIP (Fig. 1). Conversion of iOTC to mature OTC by MIP was inhibited markedly by 20 μM peptide OTC(25-34), and almost completely by a 50 μM concentration of this peptide. On the other hand, mature OTC formation was not affected by 50 μM or 100 μM peptide OTC(31-40) (Fig. 1).

Fig. 1. The octapeptide and the two amino-terminal residues of mature OTC inhibit processing of iOTC to mature OTC. In vitro-translated, [35S]methionine-labeled pOTC was incubated with purified rat liver MPP, or MPP plus MIP, as indicated, as described under "Experimental Procedures." In the absence of MPP, pOTC was processed to precursor OTC, mature OTC, and intermediate OTC as indicated by SDS/PAGE and fluorography. Flanking sequences are indicated.
Because both peptides contain the normal MIP cleavage site between glutamine 32 and asparagine 33 of the pOTC sequence, it is possible that MIP inhibition by OTC(25–34) is not simply caused by an excess of processing sites provided by this peptide. To test whether the octapeptide alone, not the sequence, it is possible that MIP inhibition by OTC(25–34) is not simply caused by an excess of processing sites provided by the peptide, we synthesized a peptide corresponding to residues 25–32 of the pOTC sequence (i.e., residues 1–8 of the octapeptide) (see Fig. 2 for peptide sequence). Processing of pOTC to iOTC by MPP was not inhibited by 50 μM or 100 μM peptide OTC(25–32) (Fig. 2).

MIP inhibition by this peptide was analyzed separately using a fraction enriched in MIP activity and free of MPP activity (Isaya et al., 1991) and construct Met-(OTC26–32)-OTC as substrate (such a construct is indicated in Fig. 2 and below as Met-iOTC). This construct is translated from a methionine that substitutes for the phenylalanine normally present at the amino terminus of iOTC. We have previously shown that this artificial intermediate is processed by purified MIP to mature OTC regardless of the presence of MPP (Isaya et al., 1991). Conversion of Met-iOTC to mature OTC was inhibited in the presence of increasing concentrations of peptide OTC(25–32), from 0.5 μM to 5 μM, and inhibition was almost complete at 20 μM (Fig. 2).

To analyze whether a specific octapeptide is required for MIP inhibition or whether any 8 amino-terminal residues may be recognized by this peptidase, Met-iOTC was incubated with MIP in the presence of 100 μM peptide OTC(31–40), random octapeptide (A), and random decapeptide (B) (see Fig. 2 for peptide sequences). These peptides were chosen because their amino acid composition is similar to that of the intermediate octapeptides, although peptides A and B do not contain either phenylalanine, leucine, or isoleucine at the amino terminus, nor serine, threonine, or glycine at position 4, while in peptide OTC(31–40), a leucine is present at the amino terminus. No significant inhibition was observed with any of these peptides (Fig. 2).

By densitometry of the fluorogram shown in Fig. 2, we determined the amount of iOTC formed by MPP, as well as the amount of mature OTC formed by MIP in the presence of the various peptides, and expressed it, respectively, as the percentage of iOTC or mature OTC formed in the absence of peptides. At 50 μM and 100 μM peptide OTC(25–32), conversion of pOTC to iOTC by MPP was 132% and 91%, respectively, relative to formation of iOTC in the absence of peptide. Conversion of Met-iOTC to mature OTC by MIP was 102% at 0.05 μM, 56% at 0.5 μM, 48% at 2 μM, 36% at 5 μM, and 0.8% at 20 μM peptide OTC(25–32), relative to formation of mature OTC in the absence of peptide. Conversion of Met-iOTC to mature OTC was 68% in the presence of 100 μM peptide OTC(31–40), 87% in the presence of 100 μM peptide A, and 75% in the presence of 100 μM peptide B, relative to formation of mature OTC in the absence of peptides.

These results indicate that the OTC octapeptide is specifically recognized by MIP, regardless of the presence of the carboxyl-terminal half of the MIP cleavage site.

The Amino-terminal Hydrophobic Amino Acid Is Essential for Recognition of the Octapeptide by MIP—Although the MIP cleavage site is normally present in the precursor molecule, we have shown that cleavage of the precursor by MPP is necessary for subsequent cleavage of the intermediate by MIP (Sztul et al., 1987). This suggests that the octapeptide may have to be present at the amino terminus of the intermediate to be recognized by MIP. Because the first amino acid in the octapeptide becomes the amino-terminal residue upon cleavage of the precursor by MPP, and because only certain hydrophobic amino acids are found at the amino terminus of the intermediate octapeptides, the amino terminus of the octapeptide may be important for recognition by MIP.

We synthesized a peptide corresponding to residues 26–32 of the pOTC sequence (i.e., residues 2–8 of the octapeptide), not including phenylalanine 25, normally at the amino terminus of the octapeptide (see Fig. 3 for peptide sequence). Peptide OTC(26–32) did not inhibit processing of Met-iOTC to mature OTC (Fig. 3). Densitometry of the fluorogram shown in Fig. 3 indicated that conversion of Met-iOTC to mature OTC by MIP was 92% at 5 μM, 89% at 20 μM, 90% at 50 μM, and 93% at 100 μM peptide OTC(26–32), relative to formation of mature OTC in the absence of peptide.

Either the absence of the amino-terminal phenylalanine and/or the presence of seven rather than eight amino acids in peptide OTC(26–32) may be responsible for the lack of MIP inhibition by this peptide. To determine whether the amino-terminal hydrophobic residue or the length of the peptide is more important for recognition by MIP, we synthesized a peptide corresponding to residues 25–31 of the pOTC sequence (i.e., residues 1–7 of the octapeptide), in which the amino-terminal phenylalanine was deleted (see Fig. 3 for peptide sequence). Peptide OTC(25–31) inhibited conversion of Met-iOTC to mature OTC by MIP, although higher concentrations of peptide were required. Densitometry of the fluorogram shown in Fig. 3 indicated that conversion of Met-iOTC to mature OTC by MIP was 89% at 5 μM, 56% at 20 μM, 45% at...
50 μM, and 25% at 100 μM peptide OTC(25–31), relative to formation of mature OTC in the absence of peptide. Therefore, the amino-terminal phenylalanine represents an essential element for recognition of the OTC octapeptide by MIP, while the length of the peptide plays a secondary role.

Because large hydrophobic amino acids are found at the amino terminus of the octapeptides, and because methionine, another of the largest hydrophobic amino acids, can efficiently substitute for phenylalanine in Met-iOTC, either the hydrophobicity and/or the size of the amino acid at the octapeptide amino terminus may be important for MIP recognition. We synthesized a peptide corresponding to the OTC octapeptide in which tyrosine was substituted for the amino-terminal methionine, and because methionine, than those required for inhibition by OTC(25–32). Densitometry of the fluorogram shown in Fig. 3 indicated that conversion of Met-iOTC to mature OTC by MIP was 78% at 5 μM, 53% at 20 μM, 45% at 50 μM, and 25% at 100 μM peptide OTC(25–32), relative to formation of mature OTC in the absence of peptide. Although the hydrophobicity of phenylalanine is not much greater than that of tyrosine, the presence of a hydrophilic hydroxyl group on the phenolic ring of tyrosine appears to be sufficient for decreased recognition of the octapeptide by MIP.

To better quantitate and compare inhibition of MIP by the various peptides, we performed three independent determinations of MIP inhibition in the presence of different concentrations of each peptide. By densitometry of fluorograms, we determined the amount of mature OTC formed by MIP in the presence of increasing concentrations of the various peptides and expressed it as percentage of mature OTC formed in the absence of peptides. The values that we obtained are shown in Table I.

**MIP Is Inhibited by Neurospora Fe/S Intermediate Octapeptide**—We performed similar experiments using pMDH as substrate (Fig. 4). This precursor was incubated with MPP and MIP in the presence of increasing concentrations of various peptides. Processing of pMDH to iMDH by MPP was not inhibited by any of the synthetic peptides tested. On the other hand, conversion of iMDH to mature MDH by MIP was inhibited by concentrations of peptide OTC(25–32) ranging from 0.5 μM to 5 μM with essentially complete inhibition at 20 μM, as we observed when iOTC (see Fig. 1) or Met-iOTC (see Fig. 2) were used as substrates. No apparent inhibition was obtained with peptide OTC(26–32) at concentrations up to 100 μM (Fig. 4), while peptide OTC(25–32) inhibited MIP at concentrations at least 10-fold higher than those required to obtain the same level of inhibition with peptide OTC(25–32). No significant inhibition of MIP activity was observed at 50 μM and 100 μM peptide OTC(31–40) and random octapeptide A. Therefore, under our experimental conditions, inhibition of MIP by the synthetic OTC octapeptide is independent of the intermediate used as substrate.

On the other hand, different concentrations of peptide may be required to obtain MIP inhibition when using a synthetic octapeptide that contains a hydrophobic residue different from phenylalanine at the amino terminus. A peptide corresponding to residues 25–32 of the Neurospora pFe/S sequence (i.e. residues 1–8 of the octapeptide) was synthesized. This octapeptide normally contains a leucine at the amino terminus (see Fig. 5 for peptide sequence). Peptide Fe/S(25–32) inhibited conversion of Met-iOTC to mature OTC by MIP. Densitometry of the fluorogram shown in Fig. 5 indicated that conversion of Met-iOTC to mature OTC by MIP was 78% at 5 μM, 53% at 20 μM, 45% at 50 μM, and essentially not detectable at 100 μM peptide Fe/S(25–32), relative to formation of mature OTC in the absence of peptide. Conversion of Met-iOTC to mature OTC was 75% in the presence of 100 μM peptide OTC(31–40), relative to formation of mature OTC in the absence of peptide. Similar results were obtained when pFe/S was used as substrate (Fig. 5). While conversion of pFe/S to iFe/S by MPP was not affected by up to 100 μM peptide Fe/S(25–32), conversion of iFe/S to mature Fe/S by MIP was 90% at 50 μM and not detectable at 100 μM concentrations of peptide.

**Amino-terminal Octapeptides Are Necessary for Cleavage of Intermediates by MIP**—To investigate the requirements for removal of the octapeptides from the intermediate proteins by MIP, we analyzed processing of two artificial intermediates, Met-(OTC26–32)-OTC (i.e. Met-iOTC) and Met-(OTC25–32)-OTC, by purified MIP in vitro. These two proteins are essentially identical, except that in Met-(OTC26–32)-OTC an initiating methionine has been substituted for the amino-terminal phenylalanine, while in Met-(OTC25–32)-OTC the methionine has been added to the amino terminus of the octapeptide in front of the phenylalanine. Therefore, relative to the mature OTC amino terminus, methionine is at position −8 in Met-(OTC26–32)-OTC, and at −9 in Met-(OTC25–32)-OTC. Wild-type pOTC and the two intermediate OTC proteins were incubated with purified MPP and MIP (Fig. 6). In the presence of MPP, pOTC was processed to iOTC (lane 2), while no proteolytic activity was observed upon incubation of pOTC with MIP alone (lane 3). Incubation of pOTC with MPP plus MIP led to conversion of iOTC to mature OTC (lane 4). Met-(OTC26–32)-OTC was not processed by MPP (lane 6), while incubation with MIP led to formation of mature OTC (lane 7), and incubation with MIP

| Peptide          | Peptide concentration |
|------------------|-----------------------|
|                  | 0 μM      | 0.05 μM  | 0.5 μM   | 2 μM       | 5 μM       | 20 μM      | 50 μM      | 100 μM     |
| OTC (25–32)      | 100       | 93 ± 15  | 61 ± 12  | 47 ± 1     | 36 ± 1     | 3 ± 2      | 12 ± 1     | 18 ± 1     |
| OTC (25–32)      | 100       | 73 ± 11  | 71 ± 15  | 47 ± 2     | 22 ± 1     | 12 ± 1     | 18 ± 1     | 18 ± 1     |
| OTC (26–32)      | 100       | 89 ± 4   | 95 ± 7   | 94 ± 12    | 86 ± 9     | 86 ± 9     | 86 ± 9     | 86 ± 9     |
| OTC (25–31)      | 100       | 92 ± 4   | 74 ± 18  | 43 ± 26    | 19 ± 8     | 19 ± 8     | 19 ± 8     | 19 ± 8     |
| OTC (31–40)      | 100       | 77 ± 10  | 77 ± 10  | 77 ± 10    | 77 ± 10    | 77 ± 10    | 77 ± 10    | 77 ± 10    |
| Random octa-pA   | 100       | 95 ± 10  | 95 ± 10  | 95 ± 10    | 95 ± 10    | 95 ± 10    | 95 ± 10    | 95 ± 10    |
| Random decap-pA  | 100       | 86 ± 14  | 86 ± 14  | 86 ± 14    | 86 ± 14    | 86 ± 14    | 86 ± 14    | 86 ± 14    |

**Table I**

**Inhibition of MIP by synthetic peptides**

Met-iOTC was incubated with purified MIP in vitro, as described under “Experimental Procedures,” in the presence of the indicated peptides. Three independent determinations were performed for each peptide at the indicated concentrations. The amounts of OTC formed by MIP from Met-iOTC in the presence of the peptides are expressed as percent of OTC formed in the absence of peptides. Values were determined by densitometry of fluorograms and expressed as mean ± S.D.
pMDH was incubated with MPP plus MIP, as indicated. Increasing concentrations of peptides OTC(25-32), OTC(26-32), and OTC(26-40) were added to different processing reactions as indicated. The products of the reactions were analyzed by SDS/PAGE, fluorography, and densitometry. The peptide corresponding to the Neurospora pFe/S octapeptide. In vitro-translated, [35S]methionine-labeled pFe/S was incubated with MPP plus MPP led to conversion of iFe/S to mature Fe/S. The reactions were analyzed by SDS/PAGE and fluorography. The peptide sequences are as indicated in Fig. 3.

pOTC and MIP cleavage sites are indicated. The asterisk marks Arg-23 in the pOTC leader, 1 marks the initiating methionine in each of the two artificial intermediates. In vitro-translated pOTC, Met-(OTC26-32)-OTC, and Met-(OTC25-32)-OTC were incubated with MPP alone (lanes 4, 8, and 12), as described under “Experimental Procedures.” The products of the reactions were analyzed directly by SDS/PAGE and fluorography.

plus MPP did not have any additional effect (lane 8). In contrast, Met-(OTC25-32)-OTC was not cleaved by MIP (lanes 11 and 12). Because neither the octapeptide nor the MIP cleavage site has been modified in this protein, and because methionine is present at the amino terminus of both constructs, we conclude that cleavage of intermediates by MIP requires that the octapeptide is presented at the amino terminus of the intermediate, such that the amino-terminal residue of the octapeptide and the amino-terminal residue of the intermediate are identical.

Structural Information in the Amino Terminus of Twice-cleaved Mature Proteins Is Also Required for Cleavage of the Intermediate by MIP—We further analyzed whether the presence of an amino-terminal octapeptide may be sufficient for MIP cleavage regardless of the structural characteristics of the mature passenger protein. Because there are data to indicate that the mature amino terminus of twice-cleaved proteins is structurally different from the mature amino terminus of once-cleaved proteins (Gavel and von Heijne, 1990; Isaya et al., 1991), we synthesized artificial intermediate peptides bearing the Met-(OTC26-32) octapeptide joined to the mature amino terminus of a twice-cleaved (pFe/S; pMDH) or a once-cleaved (pF1;β) mitochondrial protein. Wild type pFe/S and construct Met-(OTC26-32)-Fe/S were incubated with purified MPP and MIP in vitro (Fig. 7a). Incubation of pFe/S with MPP led to formation of iFe/S (lane 2), and incubation with MPP plus MIP led to conversion of iFe/S to mature Fe/S by MIP (lane 4). Construct Met-(OTC26-32)-Fe/S was processed to mature Fe/S by MIP (lane 7), similarly to the iFe/S produced during two-step processing of pFe/S. No proteolytic activity was observed upon incubation of Met-(OTC26-32)-Fe/S with MPP alone (lane 6), and incubation with MIP and MPP did not have any additional effect as compared to cleavage by MIP alone (lanes 7 and 8). Similar results were obtained with construct Met-(OTC26-32)-MDH (not shown).

On the other hand, construct Met-(OTC26-32)-F1;β was not cleaved by MIP (Fig. 7b, lane 15), as was the case for wild type proteins bearing the Met-(OTC26-32) octapeptide joined to the mature amino terminus of twice-cleaved (pFe/S; pMDH) or a once-cleaved (pF1;β) mitochondrial protein. Wild type pFe/S and construct Met-(OTC26-32)-Fe/S were incubated with purified MPP and MIP in vitro (Fig. 7a). Incubation of pFe/S with MPP led to formation of iFe/S (lane 2), and incubation with MPP plus MIP led to conversion of iFe/S to mature Fe/S by MIP (lane 4). Construct Met-(OTC26-32)-Fe/S was processed to mature Fe/S by MIP (lane 7), similarly to the iFe/S produced during two-step processing of pFe/S. No proteolytic activity was observed upon incubation of Met-(OTC26-32)-Fe/S with MPP alone (lane 6), and incubation with MIP and MPP did not have any additional effect as compared to cleavage by MIP alone (lanes 7 and 8). Similar results were obtained with construct Met-(OTC26-32)-MDH (not shown).
type pF₁₋β (lane 11). Interestingly, Met-(OTC26–32)-F₁₋β was processed by MPP to the mature-sized F₁₋β (lane 14), as observed for the wild type pF₁₋β (lane 10), and incubation with MPP plus MIP did not have any additional effect as compared to cleavage by MPP alone (lanes 12 and 16). Note that in order to increase the resolution between precursor, intermediate, and mature forms of pF₁₋β and Met-(OTC26–32)-F₁₋β, these two proteins had 245 carboxyl-terminal amino acids deleted, as described under “Experimental Procedures.” The same pattern of processing was obtained with full length pF₁₋β and Met-(OTC26–32)-F₁₋β (not shown).

**DISCUSSION**

Maturation of a number of mitochondrial precursors requires two sequential cleavages carried out by the mitochondrial processing peptidase (MPP) and the mitochondrial intermediate peptidase (MIP) in the mitochondrial matrix (Kalousek et al., 1988). Although the relationship among the precursors is not understood, they are characterized by the presence of specific octapeptides between the MPP and the MIP cleavage sites (Hendrick et al., 1989; von Heijne et al., 1989; Gavel and von Heijne, 1990). Because MPP and MIP cleave these precursors sequentially and cleavage by MIP can only follow cleavage by MPP (Ssoul et al., 1988), the octapeptide is found at the carboxyl-terminal side of the MPP cleavage site in the precursor, and at the amino-terminal side of the MIP cleavage site upon formation of the intermediate by MPP. This implies that distinct features in the octapeptide may be required for cleavage of the precursor by MPP and of the intermediate by MIP, respectively.

We have shown previously that the requirement of an octapeptide between the MPP cleavage site and the mature amino terminus of twice-cleaved precursors relates to specific structural characteristics of this subset of mitochondrial proteins (Isaya et al., 1991). The mature amino terminus of twice-cleaved precursors is structurally incompatible with cleavage by MPP, and intermediate octapeptides are required to provide compatible MPP cleavage sites in these precursors.

In the present study we demonstrate that the octapeptide is required at the amino terminus of the intermediate to provide a specific signal for MIP and an optimized MIP processing site. We show that synthetic octapeptides, corresponding to the intermediate octapeptides normally found in human pOTC and Neurospora pFe/S, inhibit MIP activity in *vivo*. This result indicates that the octapeptide can be recognized by MIP regardless of the presence of the corresponding mature protein and of the carboxyl-terminal half of the MIP cleavage site as well. Synthetic octapeptides interact with MIP at a site that is apparently crucial for processing activity, probably competing for this site with the octapeptide present at the amino terminus of the intermediate used as substrate.

On the other hand, MPP activity is not affected by concentrations of octapeptide 5-fold higher than those required to obtain complete inhibition of MIP activity, supporting our previous conclusion that the octapeptide is not recognized by MPP *per se* but as a compatible structure within the precursor molecule.

The amino-terminal hydrophobic residue is essential for recognition of synthetic octapeptides by MIP. This is not surprising considering the fact that the amino terminus of the octapeptide becomes the amino terminus of the intermediate upon cleavage of the precursor by MPP and that only certain large hydrophobic amino acids are found at the octapeptide amino terminus (Hendrick et al., 1989; von Heijne et al., 1989). Because substitution of tyrosine for phenylalanine considerably decreases the affinity of MIP for the OTC octapeptide, the hydrophobicity of the amino-terminal residue in the octapeptide appears to be a critical feature for interaction with the peptidase.

Deletion of the amino-terminal phenylalanine completely abolishes inhibition of MIP by the OTC octapeptide. On the other hand, deletion of the carboxyl-terminal glutamine still allows recognition of the synthetic peptide by MIP, indicating that the amino terminus of the octapeptide is more crucial for recognition than the residues that follow. This is not to say that any peptide bearing a hydrophobic residue at the amino terminus may be recognized by MIP. Control peptide OTC(31–40) does not inhibit MIP despite the presence of a leucine residue at the amino terminus, in contrast to MIP inhibition by peptide Fe/S(25–32), in which the amino-terminal leucine is followed by the rest of the Fe/S octapeptide. Therefore, the role of residues 2–8 of the octapeptide appears to be important for recognition by MIP although subordinate to the presence of the hydrophobic amino acid at position 1.

We have shown previously that the amino-terminal residues of the octapeptide are sufficient for sequential cleavage of pOTC by MPP and MIP (Isaya et al., 1991). When the 2 carboxyl-terminal residues of the octapeptide are deleted, however, cleavage by MPP generates the normal iOTC amino terminus, while sequential cleavage by MIP occurs at position +2 relative to the normal mature OTC amino terminus, such that precisely 8 residues are removed from the amino terminus of the intermediate. Therefore, although the presence of the amino-terminal hydrophobic residue may still allow recognition of synthetic octapeptides whose carboxyl termini have been deleted, cleavage of the intermediate by MIP seems to require the presence of a complete octapeptide on the amino-terminal half of the MIP cleavage site.

Our data indicate further that the octapeptide only is allowed upstream from the MIP cleavage site. When methionine is added before the amino terminus of the octapeptide in construct Met-(OTC25–32)-OTC, the resulting intermediate is not processed by MIP. However, when methionine is substituted for phenylalanine, such that the amino terminus of the octapeptide and the amino terminus of the intermediate are identical, the resulting intermediate is cleaved by MIP to mature OTC. Correct spacing between the amino terminus of the intermediate and the mature protein amino terminus may be crucial for cleavage of intermediates by MIP, and amino-terminal octapeptides may function to supply optimized processing sites in these proteins.

There are data to indicate that the mature amino terminus of twice-cleaved proteins is structurally different from the mature amino terminus of once-cleaved proteins (Gavel and von Heijne, 1990; Isaya et al., 1991). The structure in the mature amino terminus of twice-cleaved proteins which is not compatible with MPP cleavage appears to be specifically recognized by MIP. On the other hand, the presence of an amino-terminal octapeptide is not alone sufficient to direct MIP cleavage regardless of the structural characteristics of the passenger protein. The striking difference in processing between intermediates carrying the Met-(OTC26–32)-octapeptide joined, respectively, to the mature amino terminus of twice- or once-cleaved precursors, cannot be explained by the presence of different consensus sequences in the mature amino termini of these two subsets of proteins. A consensus sequence, in fact, has not been identified in the carboxyl-terminal halves of either the MPP or the MIP cleavage sites (Hendrick et al., 1989; von Heijne et al., 1989; Gavel and von Heijne, 1990). Therefore, structural features in the mature amino terminus of once- and twice-cleaved precursors prob-
ably direct cleavage of these proteins by MPP or MIP, respectively. Amino-terminal octapeptides are necessary and sufficient for recognition of intermediates by MIP, but can only be cleaved within the structural context of a twice-cleaved protein.

These data provide further evidence that those mitochondrial enzymes whose biogenesis involves cleavage by MIP constitute a functionally distinct family of proteins. We have shown that two-step processing responds to specific structural characteristics of such proteins. We are currently investigating the biological correlation among these proteins and the cleavage catalyzed by MIP.

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