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Characterization of the Mitochondrial Processing Peptidase of *Neurospora crassa**

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The mitochondrial processing peptidase (MPP) of *Neurospora crassa* is constituted by an α- and a β-subunit. We have purified α-MPP after expression in *Escherichia coli* while β-MPP was purified from mitochondria. A fusion protein between precytochrome b_2 and mouse dihydrofolate reductase was expressed in *E. coli*, and the purified protein was used as substrate for MPP. Both subunits of MPP are required for processing. MPP removes the matrix targeting signal of cytochrome b_2 by a single cut, and the resulting presequence peptide is 31 amino acid residues in length. It acts as a competitive inhibitor of processing but has a ~30-fold lower affinity for MPP than the preprotein. Competition assays show that MPP recognizes the COOH-terminal portion of the presequence of cytochrome b_2 rather than the NH_2-terminal part which has the potential to form an amphiphilic helix. Substitution of arginine in position −2 of the matrix targeting sequence of cytochrome b_2 prevents processing but not import of a chimeric precursor. Substitution of the tyrosyl residue in position +1 also prevents processing, indicating that MPP interacts with sequences COOH-terminal to the cleavage site. Noncleavable preprotein is still recognized by MPP. Our data suggest that processing peptidase and import machinery recognize distinct structural elements in preproteins which, however, can be overlapping.

The majority of nuclear encoded mitochondrial proteins are synthesized with amino-terminal presequences (for reviews, see Hartl and Neupert (1990) and Attardi and Schatz (1988)). These presequences target the preproteins to the mitochondrial matrix where they are proteolytically removed by the *mitochondrial processing peptidase* (MPP)[1] (Böhni *et al.*, 1980; Schmidt *et al.*, 1984; Emr *et al.*, 1986, Kalousek *et al.*, 1988). MPP was initially purified from *Neurospora crassa* (Hawlitschek *et al.*, 1988) and *Saccharomyces cerevisiae* (Yang *et al.*, 1988) and subsequently from rat liver (Ou *et al.*, 1989; Kleiber *et al.*, 1990) and from potato tubers (Braun *et al.*, 1992). The *N. crassa* peptidase is constituted by two polypeptides α-MPP[2] and β-MPP with molecular masses of 57 and 52 kDa, respectively. The two polypeptides are structurally related but functionally distinct (Pollock *et al.*, 1988; Schulte *et al.*, 1989). Neurospora β-MPP is identical to the core I protein of the ubiquinol cytochrome *c* reductase (bc_1-complex). The core II protein is related to α- and β-MPP, but does not have processing activity. In yeast α-MPP is encoded by the MAS2 gene (Pollock *et al.*, 1988; Jensen and Yaffe, 1988) and β-MPP is the product of the MAS1 gene (Witte *et al.*, 1988). The two proteins are related to but distinct from the core proteins of the bc_1-complex. In mitochondria from fungi as well as from rat liver the processing activity is found soluble in the matrix. By contrast, all detectable processing activity in mitochondria from potato tubers is membrane bound and associated with the bc_1-complex. However, it is not yet known which of the three core-like proteins constitute the active peptidase (Braun *et al.*, 1992).

The presequences which are removed by MPP are heterogeneous in length and do not show significant sequence homology. They are generally rich in basic and hydroxylated amino acids. The molecular basis for presequence specific interactions is not yet understood. In particular it is not known, whether the information for recognition by the import machinery and by the processing peptidase is contained in identical or distinct structural elements of the presequence. Stretches within mitochondrial presequences have the potential to form amphiphilic α-helical structures (von Heijne, 1986; Roise *et al.*, 1986). Several lines of evidence suggest that these regions are crucial for targeting of preprotein to the mitochondrial matrix. Amino acid substitutions which reduce or destroy the amphiphilic potential of a presequence strongly affect or prevent import of the preproteins such as the β-subunit of the F_1-ATPase ($pF_1\beta$), the ornithine transcarbamoylase (pOTC), and cytochrome oxidase subunit IV (pCOX IV) (Emr *et al.*, 1986; Horwich *et al.*, 1987; Hurt *et al.*, 1987). The site of processing of preproteins by MPP is often found at the COOH-terminal border of the putative amphiphilic structure. A characteristic feature of the processing site is the presence of an arginyl residue in position −2 with respect to the cleavage site. This arginine is conserved in about 70% of mitochondrial preproteins from different species (Nicholson and Neupert, 1988; Hendrik *et al.*, 1989; Hartl *et al.*, 1989; Gavell and von Heijne, 1990; Arretz *et al.*, 1991). A substitution of the −2 arginine in the presequence of pOTC prevents import of the precursor into isolated mitochondria and processing of the precursor by a mitochondrial extract (Horwich *et al.*, 1986).

Here we describe the large scale preparation of both components of the mitochondrial processing peptidase of *N. crassa*. We also report the preparation of a suitable preprotein substrate by expression in *Escherichia coli*. Using purified pepti-

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[1] The abbreviations used are: MPP, mitochondrial processing peptidase; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; TMPD, *N,N,N′₂N′*-tetramethyl-*p*-phenylenediammonium.

[2] In accordance with a unification of the nomenclature for mitochondrial peptidases (Kalousek *et al.*, 1993) the large subunit of *N. crassa* MPP (formerly MPP) is called α-MPP and the small subunit (formerly PEP) is called β-MPP.

dase and preprotein we established an *in vitro* assay system for processing. We show that both components of the peptidase are required for catalytic activity. MPP removes the matrix targeting signal of precytochrome b_2 by a single cut. The resulting presequence peptide is recognized by the peptidase with reduced affinity and acts as competitive inhibitor of the processing reaction. A peptide derived from the COOH-terminal portion of the cytochrome b_2 presequence competes for processing of the preprotein, but not a peptide derived from the NH_2 terminus which has the potential to form an amphiphilic helix. We show that the substitution of the conserved arginyl residue in position −2 from the cleavage site and of the tyrosyl residue in position +1 prevent processing of the preprotein while import into mitochondria is not affected. Non-cleavable preprotein is still recognized by MPP.

Our data suggest that import machinery and processing peptidase recognize different features in the presequence. These different structural elements are not mutually exclusive.

EXPERIMENTAL PROCEDURES

Expression and Purification of Mature α-MPP—To express the mature form of α-MPP we replaced by polymerase chain reaction mutagenesis the leucine codon corresponding to the mature NH_2 terminus of α-MPP by an ATG codon. As a polymerase chain reaction template we used pGEM3 DNA carrying a full-length preMPP-cDNA inserted in a SP6 promoter orientation. The upstream oligonucleotide for the polymerase chain reaction was GGGCATATGGCTACGAGAGCCGCTGCTGTC which contained an *Nde*I site overlapping the ATG codon. As downstream primer we used an oligonucleotide specific to the T7-promotor. The resulting DNA fragment was cleaved with *Nde*I and *Eco*RI and subcloned into the expression vector pJLA503 (Schauder *et al.*, 1987) yielding pRM. Positive clones were verified by sequencing.

pRM was transformed into *E. coli* Ec538. For induction of α-MPP a culture which was grown in LB (100 mg/ml ampicillin) at 30 °C to OD_{578} = 0.5 was shifted 42 °C. After 2 h cells were harvested by centrifugation and resuspended in 0.04 volume of 30 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. After mixing for 15 min, cells were pelleted at 12,000 × *g* (Sorvall JA-10 rotor) and resuspended in 30 mM Tris/HCl, pH 7.5, 1 mM $MnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 1.25% Triton X-100. Cells were broken by sonication (Branson sonifier, 10 pulses of 10 s, setting 8 and 50% duty). The debris was removed by centrifugation (60 min, 12,000 × *g*, JA10 rotor). The supernatant was loaded on a 2.5 × 35-cm DEAE-cellulose column equilibrated with buffer A (30 mM Tris/HCl, pH 7.5, 0.5% Triton X-100, 10% glycerol). The column was washed with 150 ml of buffer A and eluted in buffer A with a linear gradient from 0 to 150 mM NaCl at a flow rate of 2 ml/min. Fractions containing α-MPP (110–115 mM NaCl) were pooled and loaded on a 1.6 × 3.0-cm hydroxyapatite column equilibrated with buffer B (30 mM MOPS/KOH, pH 7.1, 50 mM NaCl). The column was washed with 60 ml of buffer B and chromatographed in buffer B at a flow rate of 0.75 ml/min with a 500-ml gradient from 0 to 200 mM sodium phosphate, pH 7.1. α-MPP eluted at 140 mM sodium phosphate. Fractions containing α-MPP were pooled, dialyzed against 30 mM MOPS/KOH, pH 7.1, and concentrated with PEG-8000. All chromatography steps were carried out at 4 °C and monitored by SDS-PAGE and Western blotting (Burnette, 1981). Immune decoration was carried out with a rabbit anti-α-MPP serum and visualized using goat anti-rabbit IgG coupled to alkaline phosphatase (Blake *et al.*, 1984).

Purification of β-MPP from Mitochondrial Membranes—Mitochondria from *N. crassa* hyphae were isolated according to Sebald *et al.* (1979) and broken by sonication. Mitochondrial membranes were collected by ultracentrifugation (Ti70 rotor, 90,000 × *g*), resuspended in 30 mM MOPS/KOH, pH 7.1 (5 mg of protein/ml according to Bradford (1976)), and solubilized in 1.5% Triton X-100. After a clarifying centrifugation (JA20 rotor, 60 min, 15.000 × *g*) the supernatant was loaded on a 2.5 × 35-cm DEAE-cellulose column equilibrated with buffer C (30 mM Tris/HCl, pH 7.5, 0.5% Triton X-100). The column was washed with 150 ml of buffer C and eluted with a linear gradient (850 ml) from 0 to 300 mM NaCl (flow rate of 2 ml/min). β-MPP eluted at 90–120 mM NaCl.

Fractions containing β-MPP were pooled and loaded on a 1.6 × 30-cm hydroxyapatite column equilibrated with buffer B. The column was washed with 60 ml of buffer B and chromatographed at a flow rate of 0.75 ml/min with a 500-ml gradient from 0 to 200 mM sodium phosphate, pH 7.1. β-MPP eluted in complex with the core II protein at 140 mM sodium phosphate.

The pooled fractions were dialyzed against 30 mM Tris/HCl, pH 8.4, and loaded on a 6 × 11-cm Q-Sepharose column equilibrated with 30 mM Tris/HCl, pH 8.4. The column was washed with 30 ml of buffer containing 80 mM NaCl and developed with an 80-ml gradient from 80 to 600 mM NaCl (flow rate 1 ml/min). β-MPP eluted at 280 mM NaCl. Pooled fractions were diluted to 50 mM NaCl and rechromatographed on hydroxyapatite (1.6 × 5 cm, equilibrated with buffer B). The column was washed with 60 ml of buffer B and developed at a flow rate of 0.75 ml/min with a 50-ml gradient from 0 to 120 mM sodium phosphate, pH 7.1. β-MPP eluted at 90 mM sodium phosphate.

All steps were carried out at 4 °C and monitored by SDS-PAGE and Western blotting (Burnette, 1981).

Purification of the Fusion Protein pb_2Δ19(167)-DHFR—The chimeric preprotein pb_2Δ19(167)-DHFR was expressed in *E. coli* and purified from inclusion bodies as described by Koll *et al.* (1992). The protein was solubilized in 2 ml of 30 mM Tris/HCl, pH 8.2, 7 M urea and passed through a DEAE-cellulose column (20-ml bed volume) equilibrated with 30 mM Tris/HCl, pH 8.2, 7 M urea. The preprotein pb_2Δ19(167)DHFR was collected in the flow-through fraction.

Preparation of Mitochondria and Import of Preproteins into Isolated Mitochondria—Preproteins were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine by coupled transcription/translation (Pelham and Jackson, 1976; Stüber *et al.*, 1984). Isolation of mitochondria from *N. crassa* and *S. cerevisiae* was performed as described elsewhere (Daum *et al.* 1982; Hartl *et al.*, 1987).

The import assays contained import buffer (250 mM sucrose, 30 mM KCl, 2.5 mM $MgCl_2$, 2 mM ATP, 8 mM ascorbate, 0.2 mM TMPD, 3% (w/v) bovine serum albumin, and 20 mM MOPS/KOH, pH 7.2, preprotein (in reticulocyte lysate), and isolated mitochondria (10 mg of mitochondrial protein) in a volume of 100 ml. Import was performed at 25 °C for 5–45 min. $MnCl_2$ was added to 1 mM, and the incubation was continued for 5 min to allow completion of processing of the preprotein. Inhibition of the membrane potential and protease treatment of mitochondria after import were performed as described (Hartl *et al.*, 1986).

In Vitro Processing Assay—Standard *in vitro* processing reactions were performed at 25 °C. α- and β-MPP (4 pmol each) were prewarmed to 25 °C in 95 μl of 30 mM Tris/HCl, pH 7.7, 0.1 mM dithiothreitol, 0.1 mM $MnCl_2$. The reaction was started by addition of 200 pmol of pb_2Δ19(167)-DHFR in 5 μl of 7 M urea, 30 mM Tris/HCl, pH 8.2. The processing reaction was stopped by addition of 10 μl of Laemmli buffer (4-fold concentrated), 1% Triton X-100. Processing products were analyzed by SDS-PAGE and quantified by densitometry (Pharmacia LKB Biotechnology Inc.).

RESULTS

Purification of the Mitochondrial Processing Peptidase and Preprotein—To study processing of mitochondrial preprotein we established an *in vitro* assay using purified MPP. In *N. crassa* α-MPP comprises about 0.03% of the mitochondrial protein, and purification yields only minor amounts of protein (Hawlitschek *et al.*, 1988). Therefore, by site-directed mutagenesis of the cDNA we replaced the leucine codon at the mature NH_2 terminus of α-MPP by a methionine codon and subcloned the cDNA coding for mature α-MPP into the heat inducible expression vector pJLA 503 (Schauder *et al.*, 1987). After heat induction of a culture of the transformed *E. coli* strain, α-MPP was purified from the fraction of soluble protein by chromatography on DEAE-cellulose and hydroxyapatite. The purification procedure yielded about 2 mg of α-MPP per liter of *E. coli* culture (Fig. 1*A*).

For purification of β-MPP which is identical to the core I protein of the bc_1-complex we used mitochondrial membranes of *N. crassa* since the membrane-derived β-subunit is much more abundant than the soluble form. Membranes were solubilized in Triton X-100 and the core I-core II complex of the cytochrome *c* reductase was enriched by chromatography on DEAE-cellulose and hydroxyapatite. Subsequently β-MPP was dissociated from the core II protein by dialysis against a low ionic strength buffer and purified to homogeneity by chromatography on Q-Sepharose and hydroxyapatite (Fig. 1*B*).

To find a suitable substrate for processing by MPP we tested a series of natural preproteins (data not shown) as well as a set of chimeric proteins derived from the precursor of cytochrome

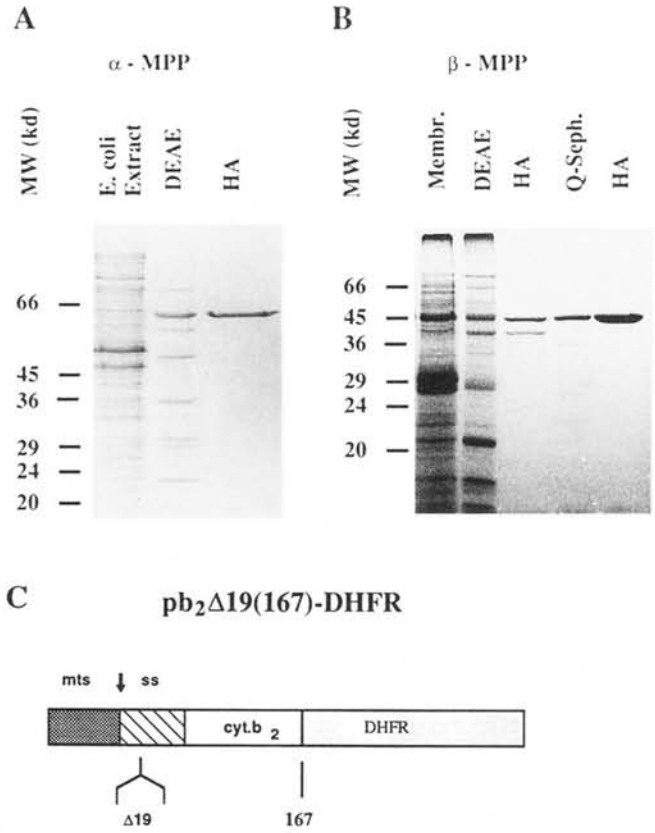

FIG. 1. **Purification of *N. crassa* α- and β-MPP and structure of chimeric preprotein.** SDS-PAGE analysis of fractions along the purification of α- and β-MPP. *A*, the mature form of *N. crassa* α-MPP was expressed in *E. coli* as described under "Experimental Procedures." The protein was purified from an extract of *E. coli* by chromatography on DEAE-cellulose and hydroxyapatite (*HA*). *B*, *N. crassa* β-MPP (core I) was purified in complex with the core II protein from Triton X-100-solubilized mitochondrial membranes (*Membr.*) by chromatography on DEAE-cellulose and hydroxyapatite. Fractions containing the core complex were dialyzed against low ionic strength buffer and subjected to chromatography on Q-Sepharose and hydroxyapatite. *C*, the structure of the chimeric preprotein $pb_2\Delta19(167)$-DHFR (Hartl *et al.*, 1986; Koll *et al.*, 1992) is schematically outlined. The NH_2-terminal portion (up to amino acid residue 167) of the precursor of *S. cerevisiae* cytochrome b_2 is fused to the mouse DHFR protein. The MPP cleavage site after the matrix targeting signal (*mts*) is indicated by an *arrow*; *Δ19* indicates the deletion of amino acid residues 47 through 65 in the sorting signal (*ss*) for the intermembrane space.

b_2 (Table I). Radiolabeled preproteins were synthesized in reticulocyte lysate and incubated for 30 min with purified MPP. Not all preproteins were competent for processing *in vitro*, although they were completely processed by MPP subsequent to import into isolated mitochondria from *N. crassa*. This indicates that presequences are not generally exposed in such a manner that MPP has full access.

When preproteins which were incompetent for processing in lysate were denatured in 7 M urea and diluted into the assay they did not become competent for processing (data not shown), while precursors such $pb_2\Delta19(167)$-DHFR were processed under the same conditions. We chose $pb_2\Delta19(167)$-DHFR, which is schematically outlined in Fig. 1*C*, as a standard substrate since it was processed most efficiently among all precursors tested. The cDNA coding for $pb_2\Delta19(167)$-DHFR was subcloned into the expression vector pUHE 73 (Bujard *et al.*, 1987). Expression in *E. coli* cells harboring the plasmid was induced with isopropyl-1-β-galactopyranoside and $pb_2\Delta19(167)$-DHFR was purified from inclusion bodies as described previously (Koll *et al.*, 1992). After solubilization of the inclusion bodies in 7 M urea SDS-PAGE resolved a single protein band (data not shown).

TABLE I
Efficiency of processing of chimeric preproteins

^{35}S-Labelled fusion proteins between NH_2-terminal portions of cytochrome b_2 and the mouse DHFR protein were synthesized in reticulocyte lysate. The length of the segment of precytochrome b_2 is given in parenthesis. $pb_2\Delta19(167)$-DHFR is described in Fig. 1*C*. Left column: the preproteins were incubated for 30 min at 25 °C with 0.01 μM MPP in 30 mM Tris/HCl, pH 7.7, 0.1 mM DTT, 0.1 mM $MnCl_2$, 0.1% Triton X-100, and 3% reticulocyte lysate. Processing was monitored by SDS-PAGE and fluorography: –, no processing; (+), partial processing; +, complete processing. Right column: radiolabeled preproteins were incubated for 5 min at 25 °C with *N. crassa* mitochondria as described under "Experimental Procedures." Under these conditions the majority of the imported cytochrome b_2 derivative was processed to the intermediate sized form and not to the mature protein (data not shown). Radiolabeled proteins were analyzed by SDS-PAGE and fluorography: +, formation of intermediate sized form resistant to proteinase K.

Precursor	Processing	
	By purified MPP	After import
pb_2(47)-DHFR	–	+
pb_2(55)-DHFR	–	+
pb_2(65)-DHFR	–	+
pb_2(76)-DHFR	–	+
pb_2(167)-DHFR	(+)	+
pb_2(331)-DHFR	(+)	+
$pb_2\Delta19(167)$-DHFR	+	+

Subsequently an anion exchange chromatography on DEAE-cellulose was performed, and the pure preprotein was collected in the flow-through fraction.

When the preprotein was diluted out of the denaturant it was efficiently processed by purified MPP. The anion exchange chromatography was crucial for the competence of the substrate for processing (data not shown). The purification of $pb_2\Delta19(167)$-DHFR yielded approximately 200 mg of preprotein per liter *E. coli* culture.

Characterization of the Processing Activity of MPP—For processing of chemical quantities of $pb_2\Delta19(167)$-DHFR the chimeric preprotein had to be diluted out of 7 M urea. Urea did not severely affect the processing reaction over a broad concentration range (Fig. 2*A*). Processing was optimal at 300 to 700 mM urea and decreased at lower and higher concentrations of the denaturant. The dependence of the processing reaction on pH and temperature is shown in Fig. 2, *B* and *C*. We established standard conditions for processing at 25 °C, 350 mM urea, pH 7.7. Using 2 μM $pb_2\Delta19(167)$-DHFR and 0.04 μM α- and β-MPP in a volume of 100 μl of processing under standard conditions was half-maximal after 2 min (Fig. 2*D*).

The dependence of the kinetics of processing on the substrate concentration is shown in Fig. 3. α- and β-MPP (0.04 μM each) were incubated for 2 min with different concentrations of $pb_2\Delta19(167)$-DHFR. Analysis of kinetic data revealed an apparent K_M of 1.27 μM and a V_{max} of 0.27 μM/min. Under the assumption that the purified enzyme is fully active the molar activity is given by: $k_{cat} = (V_{max}/(\text{MPP})) = 6.75\ \text{min}^{-1}$.

Both α- and β-MPP Are Required for Processing—To characterize the catalytic activity of MPP we assayed the two subunits of the peptidase together and each of them separately for processing activity. At a concentration of 0.4 μM neither subunit by itself had detectable processing activity, while both subunits together were highly active already at 10-fold lower concentration (Fig. 4*A*). Also, with radiochemical amounts of a substrate protein the individual subunits of MPP (0.12 μM) did not exhibit processing activity (Fig. 4*B*). Thus, both α- and β-MPP are required for the enzymatic activity of the processing peptidase.

In *N. crassa* the soluble form of β-MPP is about four times more abundant than α-MPP (Hawlitschek *et al.*, 1988). Since the two polypeptides do not form a stable complex (data not shown), the molar composition of the peptidase is not known. In

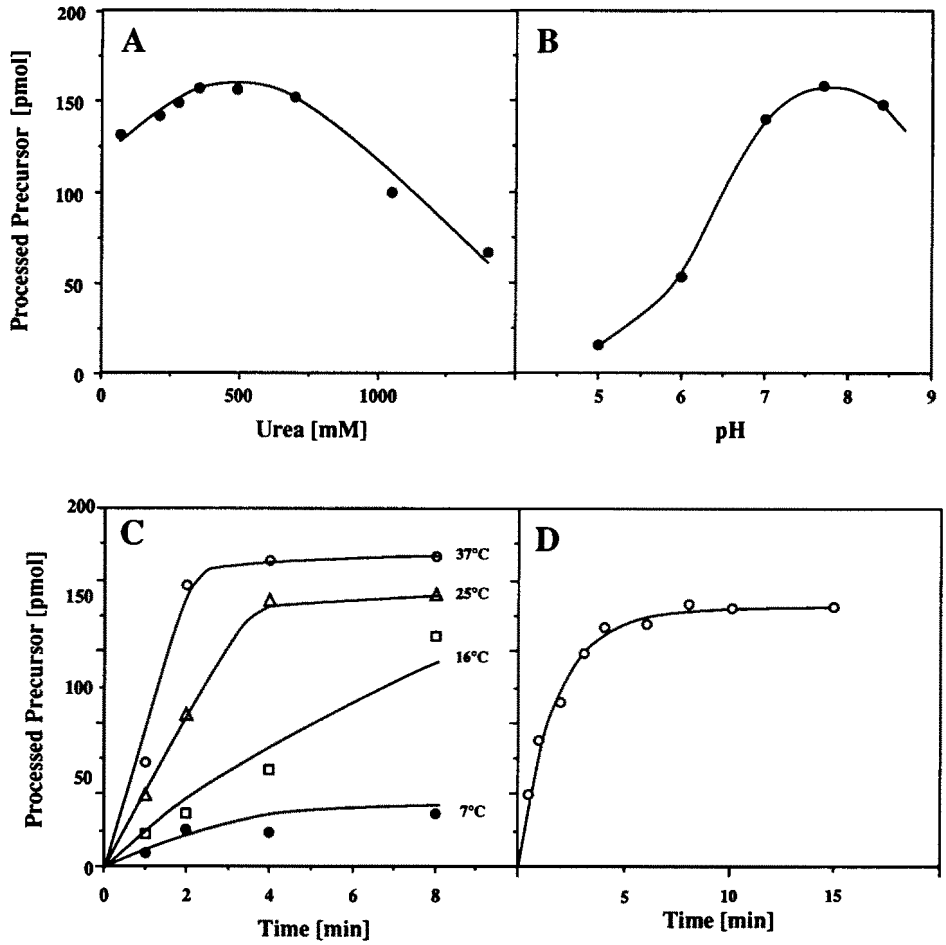

FIG. 2. **Dependence of processing of $pb_2\Delta19(167)$-DHFR on urea concentration, pH, temperature and time.** Processing reactions were carried with 2 mM $pb_2D19(167)$-DHFR and 0.04 mM MPP. Processing was monitored by SDS-PAGE and quantified by densitometry. *A*, dependence on concentration of urea. Aliquots of 1 ml containing 200 pmol of $pb_2\Delta19(167)$-DHFR in 7 M urea, 30 mM Tris/HCl, pH 8.4, were diluted into 99 μl of assay buffer containing sufficient urea to give the final concentrations indicated. *B*, pH dependence. The pH of the assay buffer was adjusted with 30 mM Bis/Tris/NaOH between pH 5.5 and 7.0 and with Tris/HCl between pH 7.0 and 8.5. *C*, temperature dependence. Processing was monitored as a function of time at the temperatures indicated. *D*, kinetics of processing under standard conditions. 200 pmol of $pb_2\Delta19(167)$-DHFR were incubated under standard conditions with 5 pmol of MPP for the time indicated. Standard assays were performed at 25 °C in 100 μl of 30 mM Tris/HCl, pH 7.7, 0.1 mM dithothreitol, 0.1 mM $MnCl_2$, and 350 mM urea.

other species the subunits of the peptidase are tightly associated and form most likely hetero-dimeric (yeast, rat) or hetero-oligomeric (potato) complexes (Yang *et al.*, 1988; Ou *et al.*, 1989; Kleiber *et al.*, 1990; Braun *et al.*, 1992).

To determine the stoichiometry of subunits of the active peptidase, processing activity of MPP was monitored at various molar ratios of α- and β-subunit. Processing activity was optimal at 1:1 molar ratio of α- and β-MPP. Up to 20-fold excess of either α- or β-MPP did not affect the processing activity. However, an increase in both subunits of the peptidase was accompanied by enhanced processing activity (Fig. 4*C*). These data indicate that at 0.04 μM peptidase interaction between α-MPP and β-MPP is not rate-limiting. The data suggest that the active peptidase is constituted by equimolar amounts of α-MPP and β-MPP.

Determination of the MPP Cleavage Site in the Presequence of Cytochrome b_2—The precursor of cytochrome b_2 is processed twice to its mature form (Guiard, 1985). The first processing step is carried out by MPP, but the cleavage site is not known. To determine the MPP cleavage site 400 pmol of $pb_2\Delta19(167)$-DHFR were processed *in vitro* by purified MPP. The processed protein was isolated and subjected to amino-terminal sequencing. The sequencing revealed **Tyr Gly Ser Thr Val** which corresponds to residues 32–38 of $pb_2\Delta19(167)$-DHFR. Thus, *N. crassa* MPP cleaves the presequence of *S. cerevisiae* cytochrome b_2 between residues 31 and 32. The penultimate amino acid in the matrix targeting sequence of cytochrome b_2 is an arginine (residue 30). Thus, the processing site in cytochrome b_2 corresponds to the consensus cleavage site.

Presequence of Cytochrome b_2 Is Removed by a Single Cleavage Step—Amino termini of processed preproteins can be determined by sequence analysis; however, sequencing does not reveal the process of maturation. In particular, it is not known whether presequences are generally removed by a single cleavage as shown for the presequence of bovine adrenodoxin (Ou et al., 1989) or by multiple cleavage events. For example, the precursor of subunit 9 of the F_0-ATPase is processed at least twice by MPP (Schmidt *et al.*, 1984). Cleavage products have not been analyzed in a system with purified components. To investigate the nature of the cleaved presequence of $pb_2\Delta19(167)$-DHFR chemical amounts of preprotein were processed *in vitro* by MPP. Subsequently, the cleavage products were analyzed by SDS-PAGE in a high Tris/urea gel and visualized by silver staining (Fig. 5). Two processing products were generated. The larger one is the processed form $ib_2\Delta19(167)$-DHFR while the low molecular weight cleavage product corresponds to the full-length presequence since it had the same electrophoretic mobility as a chemically synthesized prese-

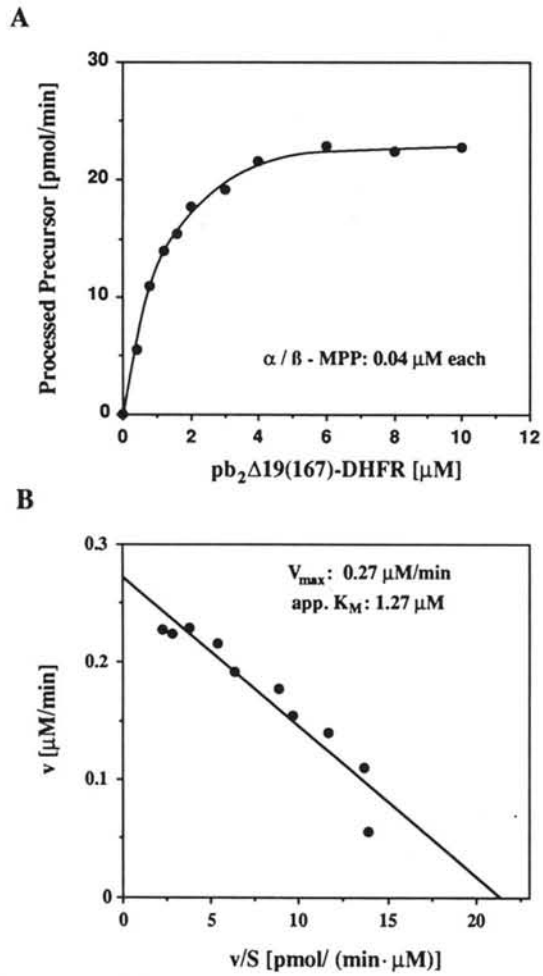

FIG. 3. **Michaelis-Menten kinetics of processing of $pb_2\Delta19(167)$-DHFR.** Processing of $pb_2\Delta19(167)$-DHFR to the intermediate sized form was carried out under standard conditions (see "Experimental Procedures") for 2 min with 0.04 μM MPP at substrate concentrations indicated. Processing was monitored by SDS-PAGE and quantified by densitometry of Coomassie Blue-stained gels. *A*, the amount of $pb_2\Delta19(167)$-DHFR processed per min is plotted *versus* substrate concentration. *B*, data are plotted according to Eadie-Hofstee. The slope of the least square fit through the data points corresponds to an apparent K_M of 1.27 μM and the *y* intercept to a V_{max} of 0.27 mM/min.

quence peptide of cytochrome b_2, $pb_2(1–31)$. Thus, the presequence of $pb_2\Delta19(167)$-DHFR is removed by a single cleavage after position 31. The presequence peptide was stable for at least 2 h in the reaction mixture and was not further degraded by MPP (data not shown).

Presequence Peptides Are Competitive Inhibitors of the Processing Reaction—We asked whether the specificity of interaction with MPP resides in the presequence peptide or whether only the intact preprotein is recognized by the peptidase. The synthetic peptide corresponding to the presequence of cytochrome b_2, $pb_2(1–31)$, was assayed for its capacity to interfere with processing of $pb_2\Delta19(167)$-DHFR. Increasing amounts of precursor were incubated with MPP in the presence and absence of the peptide (Fig. 6*A*). The presequence peptide slowed down the kinetics of processing but did not inactivate MPP. The inhibitory peptide increased the apparent K_M for processing but did not affect the maximal rate of substrate turnover, V_{max} (Fig. 6*A*, *lower panel*). This indicates that the presequence peptide acts as competitive inhibitor. In the presence of 40 μM peptide the apparent K_M for processing was 2.47 μM which is about 2-fold higher than in the absence of peptide (1.29 μM). Accordingly, the affinity of MPP for the inhibitory peptide

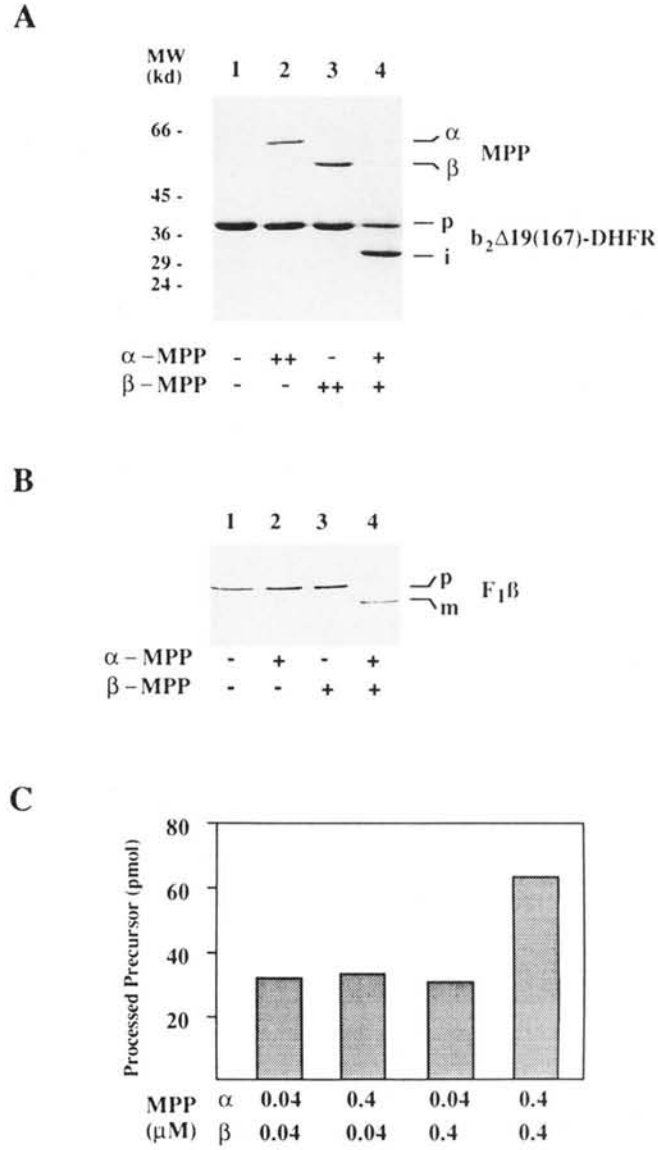

FIG. 4. **α- and β-MPP are required for processing of preprotein.** *A*, $pb_2\Delta19(167)$-DHFR (2 mM) was incubated under standard conditions for 2 min without added MPP (*lane 1*), with 0.4 μM α-MPP (*lane 2*), 0.4 μM β-MPP (*lane 3*), and 0.04 μM of each, α- and β-MPP (*lane 4*). Processing of the precursor (*p*) of $b_2\Delta19(167)$-DHFR to the intermediate sized form (*i*) was analyzed by SDS-PAGE and Coomassie Blue staining. *B*, radiochemical quantities of the precursor of the β-subunit of the F_1-ATPase ($pF_1\beta$) (*lane 1*) were incubated for 45 min with 0.04 μM α-MPP (*lane 2*), 0.04 μM β-MPP (*lane 3*), and 0.04 μM α- and β-MPP. Processing was monitored by SDS-PAGE and fluorography. Precursor (*p*) and mature (*m*) forms of $F_1\beta$ are indicated. *C* influence of the molar ratio of α- and β-MPP on processing. $pb_2\Delta19(167)$-DHFR (2 mM) was incubated for 2 min with 0.04 μM or 0.4 μM of each subunit of MPP. Processing of the precursor to the intermediate sized form was analyzed by SDS-PAGE, Coomassie Blue staining, and densitometry.

which is characterized by an apparent K_I of 37 μM (*i.e.* 1.29 × 40/(2.47 – 1.29) μM) is about 30-fold lower than for the intact preprotein.

In addition to $pb_2(1–31)$, we tested presequence peptides derived from the precursor of cytochrome c_1, $pc_1(1–36)$ and from the precursor of the β-subunit of the F_1-ATPase, $pF_1\beta(1–32)$. Each of the three peptides inhibited processing with similar efficiency while a control peptide had no influence on processing (Fig. 6*B*).

To decide whether the entire presequence of cytochrome b_2 is required for competitive inhibition we assayed two presequence-derived peptides for their ability to inhibit processing

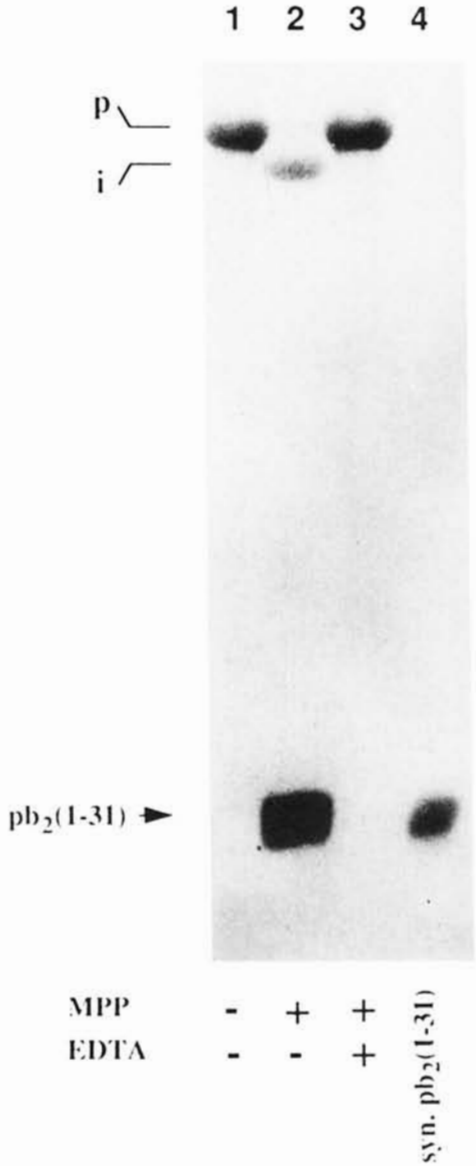

FIG. 5. **The presequence of $pb_2\Delta19(167)$-DHFR is removed by a single cut.** 400 pmol of $pb_2\Delta19(167)$-DHFR (*lane 1*) were incubated for 45 min with 4 pmol MPP under standard conditions without (*lane 2*) or with 5 mM EDTA (*lane 3*). The cleavage products were analyzed by urea-SDS-PAGE and silver staining. Precursor (*p*) and intermediate sized form (*i*) of $b_2\Delta19(167)$-DHFR are indicated. The presequence peptide of $pb_2\Delta19(167)$-DHFR is indicated by an *arrowhead*. Chemically synthesized presequence peptide $pb_2(1–31)$ was applied in *lane 4*.

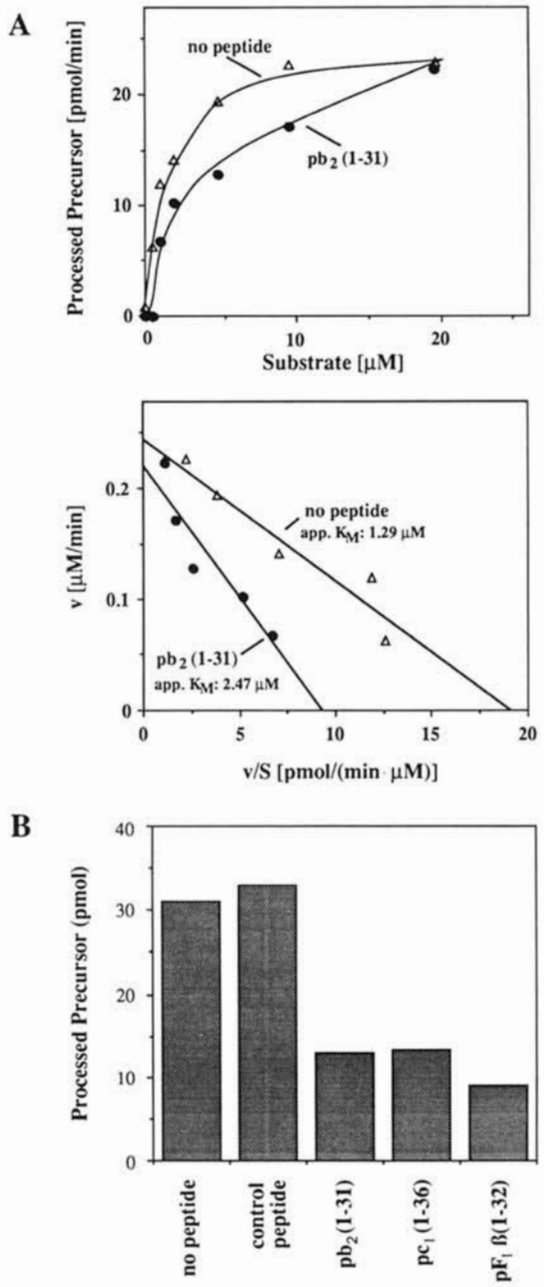

FIG. 6. **Inhibition of processing of $pb_2\Delta19(167)$-DHFR by presequence peptides.** *A*, Michaelis-Menten kinetics of processing of $pb_2\Delta19(167)$-DHFR. Indicated concentrations of $pb_2\Delta19(167)$-DHFR were incubated for 2 min with 0.04 µM MPP in the absence and presence of 40 mM presequence peptide $pb_2(1–31)$. Samples were analyzed by SDS-PAGE and densitometry. The amount of substrate protein processed per min in a 100-ml assay is plotted *versus* the substrate protein concentration. Analysis of data according to Eadie-Hofstee (*lower part*). The slopes of least square fits correspond to apparent K_M values of 1.29 µM in the absence and 2.47 µM in the presence of peptide $pb_2(1–31)$. *B*, 2 mM $pb_2\Delta19(167)$-DHFR was incubated for 2 min with MPP (0.04 µM) without or with presequence-derived peptides (100 mM). *Column 1*, no peptide added; *column 2*, control peptide derived from *N. crassa* CCHL amino acid residues 1–25; *column 3*, $pb_2(1–31)$ derived from precytochrome b_2; *column 4*, $pc_1(1–36)$ derived from precytochrome c_1; *column 5*, $pF_1(1–32)$ derived from the precursor of the β-subunit of the F_1-ATPase. The amount of intermediate sized form of $pb_2\Delta19(167)$-DHFR was determined as described above.

(Fig. 7). Peptide $pb_2(1–20)$ was derived from the NH_2-terminal portion of the presequence of cytochrome b_2 (residues 1–20) while peptide $pb_2(15–34)$ corresponded to residues 15 through 34. Both peptides were 20 residues in length. The NH_2-terminal peptide $pb_2(1–20)$ had no influence on the kinetics of processing of $pb_2\Delta19(167)$-DHFR (Fig. 7*B*). Peptide $pb_2(15–34)$, however, inhibited the cleavage reaction. Since processing at 100 µM $pb_2(15–34)$ was about half-maximal the peptide has approximately a 2-fold lower affinity for MPP than the presequence peptide $pb_2(1–31)$. Thus, MPP apparently interacts with the COOH-terminal portion of the presequence of cytochrome b_2. This portion of the presequence has no potential to form an amphiphilic α-helical structure (see Fig. 7*A*) since the calculated hydrophobic moment is not sufficiently high (*i.e.* <7; von Heijne (1986)). For the NH_2-terminal part of the presequence, by contrast, values of the hydrophobic moment are high (above 7) which is characteristic for mitochondrial targeting sequences.

Amino Acid Substitutions at Position −2 and +1 Affect Processing but Not Import of $pb_2\Delta19(167)$-DHFR—The cleavage site for MPP is frequently indicated by an arginyl residue at posi-

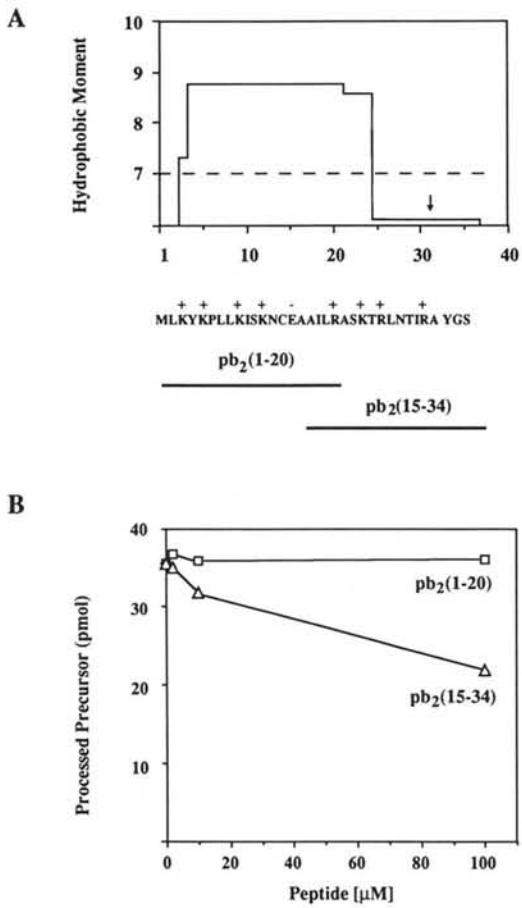

FIG. 7. **Inhibition of processing by peptides derived from the presequence of cytochrome b_2.** *A*, hydrophobic moment of the presequence of cytochrome b_2. The azpotential to form an amphiphilic α-helical structure (*Hydrophobic Moment*) was calculated for windows of 18 amino acid residues (von Heijne, 1986). Significant values of hydrophobic moment (H.M. > 7) are indicated by the *dashed line*. The sequence of the cytochrome b_2 prepeptide is shown in the *lower part*. The MPP cleavage site is indicated by an *arrow*. Peptides pb_2(1–20) and pb_2(15–34) corresponding to the NH_2- and COOH-terminal part of the presequence are *outlined*. *B*, the influence of peptides pb_2(1–20) and pb_2(15–34) on the kinetics of processing. $pb_2\Delta19(167)$-DHFR (2 μM) and and MPP (0.04 μM) were incubated for 2 min with the indicated concentrations of pb_2(1–20) and pb_2(15–34). The amount of intermediate sized form of $pb_2\Delta19(167)$-DHFR is plotted *versus* the peptide concentration.

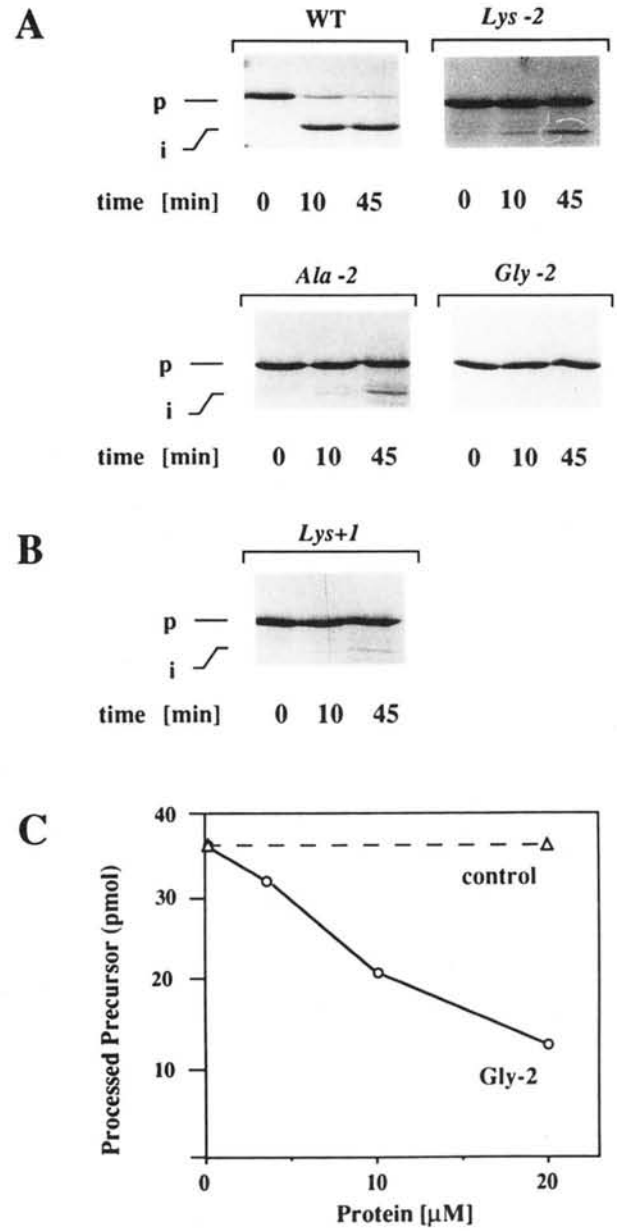

FIG. 8. **Effect of amino acid substitutions on processing of $pb_2\Delta19(167)$-DHFR.** *A*, wild-type (*WT*) and mutated $pb_2\Delta19(167)$-DHFR (1 μM) were incubated under standard conditions with MPP (0.04 mM). Reactions were terminated after the indicated time periods, and samples were analyzed by SDS-PAGE. *p*, precursor; *i*, intermediate sized form. WT protein and substitutions $Arg^{-2} \rightarrow Lys^{-2}$, $Arg^{-2} \rightarrow Ala^{-2}$, and $Arg^{-2} \rightarrow Gly^{-2}$. *B*, processing of mutated precursor carrying the substitution of $Tyr^{1} \rightarrow Lys^{-1}$. Processing was monitored as described above. *C*, inhibition of processing of $pb_2\Delta19(167)$-DHFR by the non-cleavable $Arg^{-2} \rightarrow Gly^{-2}$ mutant. $pb_2\Delta19(167)$-DHFR (2 μM) and MPP (0.04 μM) were incubated for 2 min in the presence of the non-cleavable preprotein harboring the $Arg^{-2} \rightarrow Gly^{-2}$ mutation or in the presence of bovine serum albumin (*control*) at concentrations indicated. The extent of processing of $pb_2\Delta19(167)$-DHFR is plotted as a function of protein concentration ($Arg^{-2} \rightarrow Gly^{-2}$ mutant or bovine serum albumin). Processed precursor was quantified by densitometry of Coomassie Blue-stained gels.

tion –2 (Hendrik et al., 1989; Hartl *et al.*, 1989; von Heijne *et al.*, 1989). This conserved arginine corresponds to residue 30 in the presequence of cytochrome b_2. However, the presequence of cytochrome b_2 contains further arginyl residues at positions 20 and 25 which do not define a cleavage site for MPP.

To examine the role of the -2 Arg^{-2} we substituted this amino acid by glycine, by alanine, and by lysine by site-directed mutagenesis of the respective codon in the cDNA. The resulting preproteins were expressed in *E. coli*, purified, and assayed *in vitro* for processing.

Each of the three substitutions of the –2 arginine strongly affected or prevented processing of the preproteins by MPP under standard *in vitro* conditions (Fig. 8*A*). With the mutated proteins $Arg^{-2} \rightarrow Lys^{-2}$ and $Arg^{-2} \rightarrow Ala^{-2}$ low amounts of processed precursor were detected after prolonged incubation with the peptidase. Since wild type protein was processed with a half-time of 2 min (see Fig. 2*D*) we estimate that the substitutions of arginine by alanine or lysine reduce the kinetics of processing by two orders of magnitude. Processing of the $Arg^{-2} \rightarrow Gly^{-2}$ mutant was not detectable under the conditions used (Fig. 8*A*). The non-cleavable precursor acts as inhibitor of processing of wild type precursor but is recognized by MPP with reduced affinity (Fig. 8*C*).

To investigate whether there is a requirement for specific amino acid residues COOH-terminal to the MPP cleavage site we replaced in $pb_2\Delta19(167)$-DHFR the tyrosyl residue at position +1 by a lysyl residue. This substitution leaves the entire matrix targeting sequence of precytochrome b_2 unaffected. The

TABLE II
Processing and import of wild type and mutant preproteins

Processing reaction: 200 pmol of wild-type (WT) or mutant $pb_2\Delta19(167)$-DHFR were incubated with MPP (0.04 μM) for 10 or 45 min. Processing was analyzed by SDS-PAGE, Coomassie Blue staining, and densitometry. –, no processing; (+), processing detectable (≤10%); +, complete processing (>90%). Import reaction: ^{35}S-labeled preprotein was synthesized in reticulocyte lysate and imported into mitochondria from *N. crassa* or *S. cerevisiae* ("Experimental Procedures"). Subsequent to import mitochondria were treated with proteinase K and imported proteins were analyzed by SDS-PAGE and fluorography.

Precursor $pb_2\Delta19(167)$-DHFR	Processing by purified MPP		Import into mitochondria	Processing after import
	10 min	45 min		
WT	+	+	+	+
$Arg^{-2} \rightarrow Lys^{-2}$	–	(+)	+	–
$Arg^{-2} \rightarrow Ala^{-2}$	–	(+)	+	–
$Arg^{-2} \rightarrow Gly^{-2}$	–	–	+	–
$Arg^{-2} \rightarrow Lys^{+1}$	–	(+)	+	–

$Tyr^{1} \rightarrow Lys^{1}$ mutant protein was not processed by purified MPP under standard conditions (Fig. 8*B*). After prolonged incubation with MPP some minor processing of the $Tyr^{1} \rightarrow Lys^{1}$ mutant was observed.

Radiochemical amounts of the mutant $pb_2\Delta19(167)$-DHFR preproteins were synthesized *in vitro* and assayed for import into mitochondria from *N. crassa* and *S. cerevisiae*. The mutated precursors were efficiently imported into mitochondria but none of them was processed in the mitochondrial matrix (Table II). This is exemplified in Fig. 9 for the $Arg^{-2} \rightarrow Lys^{-2}$ mutant, which is the variant preprotein that exhibited the highest level of residual processing *in vitro* (see Fig. 8*A*). Import into a proteinase K-resistant location was strictly dependent on the electrochemical potential across the mitochondrial inner membrane. However, the $Arg^{-2} \rightarrow Lys^{-2}$ mutant was not processed in the mitochondrial matrix. Even after prolonged incubation of mitochondria for up to 45 min, no intermediate sized form was detected. When wild type $pb_2\Delta19(167)$-DHFR was imported into mitochondria the precursor was efficiently processed and the intermediate sized form was resistant to proteinase K treatment of mitochondria (Fig. 9). Thus, the substitutions in position –2 and +1 abolish processing of preprotein in the mitochondrial matrix but have no detectable influence on import (Table II).

DISCUSSION

We describe an *in vitro* assay system for processing of preproteins by purified MPP from *N. crassa*. In contrast to MPP from other species the α- and the β-subunits of *N. crassa* MPP do not form a stable complex and can be isolated separately in native form. Using preparations of α- and β-MPP which were free of cross-contaminations by the corresponding second subunit we can show that both, α- and β-MPP are required for processing of preprotein. Even at μM concentrations of MPP processing of radiochemical quantities of preprotein is strictly dependent on both subunits of MPP. The low processing activity which was previously observed with α-MPP which has been immunoprecipitated from a mitochondrial extract (Hawlitschek *et al.*, 1988) is, therefore, most likely due to contamination by the β-subunit. Similarly, the extremely low processing activity which was associated with a fraction of purified MAS2 protein (yeast homologue of α-MPP) could be accounted for by contamination with the MAS1 encoded β-subunit (Geli *et al.*, 1990).

Processing of $pb_2\Delta19(167)$-DHFR by purified MPP follows Michaelis-Menten kinetics and is characterized by an apparent K_M of 1.27 μM and a turnover number of 6.75 precursor molecules/enzyme/min. Processing in mitochondria occurs along with import, and in many cases a preprotein is already processed by MPP while more COOH-terminal portions of the protein are still exposed to the cytosol. *In vitro*, by contrast, preproteins may fold into a conformation where MPP has only limited access to the presequence. In fact, we have observed significant differences in the extent of processing of a set of related preproteins which all carry the presequence of cytochrome b_2 but differ in their mature parts. Some of these proteins are not processed at all in a purified system while all of them are efficiently processed upon import into mitochondria. The K_M value determined in a purified system, therefore, does not necessarily reflect the affinity of MPP for preproteins as they emerge from the import machinery.

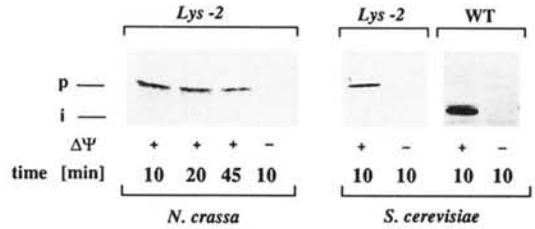

FIG. 9. **Import of wild-type and mutant preproteins into isolated mitochondria.** Mitochondria from *N. crassa* and *S. cerevisiae* were incubated with ^{35}S-labeled wild type (*WT*) and mutated preproteins harboring the Lys-2 substitution. Incubations were performed in the presence of ascorbate/TMPD (+ΔΨ) or valinomycin/KCl (–ΔΨ). After the times indicated mitochondria were treated with proteinase K and radiolabeled protein was analyzed by SDS-PAGE and fluorography. *p*, precursor; *i*, intermediate. Import assays and proteinase K treatment were performed as described under "Experimental Procedures."

The doubling time of *N. crassa* is about 3.5 h, and α-MPP comprises about 0.03% of the mitochondrial protein (Hawlitschek *et al.*, 1986). Assuming that the majority of mitochondrial proteins (*e.g.* 75%) are processed by MPP, the average rate of preprotein processing *in vivo* must be ≥12 min^{-1} (75%/0.03% per 210 min). The turnover number determined *in vitro* for processing of $pb_2\Delta19(167)$-DHFR (6.8 min^{-1}) is only 2-fold lower. Therefore, the activity of purified MPP seems to be comparable to the activity of MPP in the mitochondrial matrix characterization.

The value of k_{cat} for processing of pCOX IV-DHFR by purified MPP from *S. cerevisiae* was reported to be 1 pmol precursor/mg of enzyme/min (Yang *et al.*, 1991) which corresponds to processing of one precursor per MPP every 10 min. pCOX IV-DHFR is efficiently imported into mitochondria, and precursor does not accumulate in the matrix. Thus, it appears that in *S. cerevisiae* MPP in mitochondria is more active than in its purified state.

The matrix targeting portion of the presequence of cytochrome b_2 consists of 31 amino acids and is removed by a single cut. The presequence peptide is a competitive inhibitor of MPP but has a 30-fold lower affinity for the peptidase (K_I = 37 μM) than the preprotein. Therefore, after cleavage of a precursor a presequence peptide would rapidly dissociate from MPP and would not significantly inhibit the activity of the enzyme. This suggests that MPP may recognize a portion of the precursor which overlaps the cleavage site and that sequences COOH-terminal to the cleavage site contribute significantly to the specificity of interaction with MPP. This is supported by the amino acid substitutions in the –2 and +1 position in $pb_2\Delta19(167)$-DHFR which lead to a drastic inhibition (≥100-fold) of processing. MPP can still interact with the non-cleavable $Arg^{-2} \rightarrow Gly^{-2}$ mutant, although with a reduced affinity. This indicates that other amino acid residues contribute significantly to the specificity of interaction with the peptidase.

It has been proposed that positively charged amphiphilic α-helices function as matrix targeting signals (von Heijne, 1986; Roise *et al.*, 1986; Gavell and von Heijne, 1990). However,

it is not known whether an amphiphilic helix is recognized by MPP. In the presequence of cytochrome b_2 residues 4 through 21 have the potential to form such an amphiphilic helix while the region at the cleavage site is not predicted to be amphiphilic. Our competition assays with presequence-derived peptides indicate that MPP interacts with the region around the cleavage site rather than with the NH_2-terminal portion of the presequence of cytochrome b_2. Thus, amphiphilicity might not be a feature recognized by the peptidase. However, the potential to form such a structure in the appropriate environment does not interfere with processing as many mitochondrial preproteins become processed within or at the COOH-terminal border of the predicted amphiphilic α-helix (von Heijne, 1986; Hartl *et al.*, 1989).

Amino acid substitutions in position −2 and +1 had no influence on import of $pb_2\Delta19(167)$-DHFR into mitochondria. By contrast, a corresponding substitution of arginine −2 by glycine in the precursor of ornithine transcarbamoylase (pOTC) completely prevented processing *in vitro* and import of the precursor into isolated mitochondria (Horwich *et al.*, 1986). The differential effect of corresponding substitutions on import of $pb_2\Delta19(167)$-DHFR and of pOTC might be due to the location of the matrix targeting signal with respect to the MPP cleavage site. Thus, in the presequence of cytochrome b_2 the predicted α-helix is located at the NH_2 terminus, and amino acid substitutions in the −2 position would not affect its amphiphilic properties. In the presequence of pOTC the region with maximal hydrophobic moment overlaps the MPP cleavage site. Substitution of the arginine in position −2 by glycine should severely affect its potential to form an amphiphilic α-helix and thus destroy the matrix targeting signal.

In summary, different structural elements of mitochondrial presequences might be recognized by MPP and by the mitochondrial import machinery. These elements of recognition may be overlapping. MPP interacts with the region around the cleavage site, and portions COOH-terminal to the cleavage site may significantly contribute to this interaction. Amphiphilicity may not be recognized by the peptidase but does not interfere with processing.

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