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Biochemistry

Functional reconstitution in *Escherichia coli* of the yeast mitochondrial matrix peptidase from its two inactive subunits

(operon/coexpression/processing activity/subunit purification/*Saccharomyces cerevisiae*)

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ABSTRACT **The matrix processing peptidase from yeast (*Saccharomyces cerevisiae*) mitochondria was expressed in *Escherichia coli* via a plasmid-borne operon encoding the mature forms of the α and β subunits of the enzyme. The subunits assembled into a fully active, soluble enzyme. The mature subunits were also expressed individually. The α subunit accumulated in large amounts and was obtained at a purity of 80% after a single chromatographic step. The β-subunit-producing strain expressed an intact and a degraded form of the β subunit, both of them soluble in the cytoplasm. Extract from either the α- or the β-subunit-producing strain (S-α or S-β extract, respectively), as well as the purified α subunit, was enzymatically inactive. However, precursor cleavage activity was restored by mixing either the S-α extract or the purified α subunit with the S-β extract. The reconstituted processing activity was indistinguishable from the authentic holopeptidase.**

Most mitochondrial precursors contain amino-terminal presequences which are proteolytically removed by a matrix-localized peptidase during or after import (1–3). This peptidase has been identified in fungi, mammals, and plants (4–9). Purification of the major processing activity from *Neurospora crassa* (10), *Saccharomyces cerevisiae* (11), and rat liver (12, 13) mitochondria revealed that the enzyme is composed of two subunits of roughly similar size. However, the smaller subunit of the *N. crassa* enzyme appears to be a bifunctional protein, as 75% of the protein is a membrane-associated component of the cytochrome bc_1 complex (14). Recently, the subunits of the mitochondrial processing peptidase from potato were found to be integrated into the cytochrome bc_1 complex of the respiratory chain, with one of the subunits of the complex being the counterpart of the large subunit of the soluble peptidase (15).

The processing peptidase acts on a large number of different presequences and thus probably recognizes an unidentified three-dimensional motif. Presequences have a relatively high content in positive charges and tend to form amphiphilic α-helices (16, 17). However, recognition by the matrix peptidase requires a more stringent amino acid pattern, involving both the presequence and the amino terminus of the mature protein (18, 19). The enzyme was shown to cleave chemically synthesized peptides corresponding to matrix-targeting signal fused to eight amino acid residues of a mature protein (20). In addition, presequence peptides inhibited competitively the activity of the enzyme measured with an artificial precursor as substrate (20).

The nuclear genes encoding the matrix processing peptidase subunits have been cloned and sequenced in various species and proved to be homologous to each other (13, 15, 21–25). In the yeast *S. cerevisiae*, the genes encoding the subunits of the peptidase were termed *MAS1* or *MIF1* and *MAS2* or *MIF2* (22–24).

It has been argued that the large subunit of the processing enzyme from *N. crassa* (termed MPP) is the catalytic subunit and that the smaller subunit is a processing-enhancing protein (termed PEP) (10). However, data obtained with the yeast processing enzyme strongly suggested that neither of the subunits was active by itself (26).

According to the unification of the nomenclature of the processing peptidases, the general mitochondrial matrix peptidase is called MPP and its large (MPP or MAS2) and small (PEP or MAS1) subunits are termed the α and the β subunit, respectively (27). In this study, the yeast genes (*MAS1* and *MAS2*) encoding the mature forms of each subunit were cloned into *Escherichia coli* expression vectors and expressed individually or together in *E. coli*. Milligram amounts of the holopeptidase and of its subunits were produced by the transformed strains. We report that the α subunit of the yeast peptidase has no activity by itself. When both subunits are coexpressed in the same *E. coli* strain or when the individually expressed subunits are mixed, they assemble into an enzymatically active peptidase.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* C600 (28) was used as recipient strain for all the cloning procedures. *E. coli* W3110 (29) was used for the expression of the matrix processing peptidase and its subunits. *S. cerevisiae* strains VGA and G10, which overproduce the holoprotease and the β subunit, respectively (26), were used for the preparation of mitochondrial matrix extracts and for the isolation of mitochondrial extracts containing β subunit free of any α subunit. The expression vector pMG1 was provided by D. Baty (Laboratoire d'Ingénierie et de Dynamique des Systèmes Membranaires, Marseilles) and constructed from pJF118E/H (30). Briefly, after destruction of the single *Nde* I restriction site of pJF118E/H, a new *Nde* I restriction site followed by a multiple cloning site was introduced downstream from the *Eco*RI restriction site to give pMG1. This plasmid contains $lacI^Q$ and the *trp–lac* (*tac*) promoter, which can be derepressed by addition of isopropyl β-D-thiogalactopyranoside (IPTG).

Cloning the Genes Encoding the Mature Forms of the α and β Subunits into the *E. coli* Expression Plasmid. A 290-bp DNA fragment and a 606-bp DNA fragment extending from codon 14 to codon 121 and from codon 20 to codon 198 of the α- and β-subunit genes, respectively, were amplified by PCR. For the α-subunit gene (*MAS2*), plasmid pUC7M2 (the *MAS2* gene cloned in pUC7) was used as DNA template with the following 5′ and 3′ primers: 5′-aa-ttc-cat-atg-GCT-AGA-

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; preSu9, presequence of ATPase subunit 9; DHFR, dihydrofolate reductase.

ACC-GAT-AAT-TTT-AAG-C-3′ and 5′-C-ACA-GAA-GCC-TGA-TAC-ATC-3′. For the β-subunit gene (*MAS1*), plasmid p7.3 (the *MAS1* gene cloned in pBluescript SK from Stratagene) was used with the 5′ and 3′ primers: 5′-aa-ttc-cat-atg-TCC-TCA-CAA-ATT-CCA-GGA-ACC-3′ and 5′-GAT-CAA-CAG-CGC-CCG-CAC-C-3′. (The underlined sequences and the sequences with uppercase letters correspond to newly constructed *Nde* I restriction sites and to the coding region in the subunit genes, respectively.)

The 290-bp and 606-bp PCR products were cut by *Nde* I and *Xba* I to produce 257-bp and 161-bp fragments. Both fragments were cloned into the expression vector pMG1 after digestion with *Nde* I and *Xba* I to give pVG13 and pVG14, respectively. PCR products were sequenced by the dideoxynucleotide chain-termination method.

Full-length copies of *MAS2* and *MAS1* were obtained by DNA fragment exchange with plasmids carrying the entire *MAS* genes. A 2.3-kb *Hin*dIII fragment containing the *MAS2* gene minus its first six codons was inserted into *Hin*dIII-linearized pVG13 to give pVG15. A 2.24-kb *Xba* I–*Hin*dIII fragment from p7.3 containing the missing 3′ portion of *MAS1* was introduced in pVG14 cut with *Xba* I and *Hin*dIII, to give pVG16. pVG15 and pVG16 contain the full-length copies of the genes corresponding to the mature form of the α and β subunits under the control of the inducible *tac* promoter of pMG1.

Construction of an Operon Encoding the Mature Forms of the α and β Subunits. Two primers complementary to the *MAS1* gene were synthesized and a PCR was carried out with pVG16 as DNA template. The 3′ primer was designed to introduce, after the stop codon of the *MAS1* gene, a DNA region containing a double Shine–Dalgarno sequence, a start codon for the initiation of translation of *MAS2*, and the first eight codons of *MAS2*. This 3′ primer sequence is 3′-**G-CTT-CTT-TTT-GAC-TTG-GTT-ACT-*ATT***-gtc-ctt-tgt-cct-agg-*TAC*-CGA-TCT-TGG-CTA-TTA-AAA-TTC-GAA-AG-5′; the bold uppercase letters, the uppercase letters, the underlined lowercase letters, the italic letters, and the bold underlined upper case letters correspond to the 3′ end of the *MAS1* gene, the 5′ end of the *MAS2* gene, the Shine–Dalgarno sequences for initiation of translation, the stop and start codons of *MAS1* and *MAS2*, and the *Hin*dIII restriction site located at the 5′ end of the *MAS2* gene, respectively. The 5′ primer used for the PCR (5′-CAC-ATC-TTC-GGC-TAC-TGT-TGG-3′) was located upstream of the *Xba* I site of the β-subunit gene. The PCR produced a DNA fragment of 1302 bp. This DNA fragment was cut with *Hin*dIII and cloned into pVG16 cut with *Hin*dIII, to yield pVG17. The 2.3-kb *Hin*dIII fragment from pAC1-M2 was then cloned into pVG17 linearized with *Hin*dIII in order to clone the full-length *MAS2* gene. The resulting plasmid, pVG18, directs the synthesis of a single mRNA encoding the β and α subunits.

Preparation of Soluble Extracts of the *E. coli* Strains Producing the Matrix Processing Enzyme and Its Subunits. The *E. coli* strains W3110(pVG15), W3110(pVG17), and W3110-(pVG18) were grown in LB medium to an OD_{600} of 0.4 and induced with 1 mM IPTG for 4 hr at 30°C. Cells were washed with buffer 1 {20 mM [bis(2-hydroxyethyl)imino] tris(hydroxymethyl)methane, pH 7/50 mM NaCl/0.2 mM phenylmethanesulfonyl fluoride} and concentrated 40-fold in buffer 1. The suspension was sonicated three times for 30 sec with a microtip of a Vibra-Cell sonicator (Sonics & Materials, Danbury, CT). The extract was centrifuged at 40,000 rpm for 30 min in a Beckman 50Ti rotor. The supernatant was recovered and its protein concentration was determined.

Determination of the Amino Terminus of the α Subunit. The α subunit (2 nmol) was transferred onto a poly(vinylidene difluoride) sheet. Amino-terminal sequence analysis was performed by stepwise Edman degradation with an Applied Biosystems model 470A gas-phase sequencer.

Purification of the α Subunit from W3110(pVG15). A 400-ml culture of strain W3110(pVG15) was grown in LB medium to an OD_{600} of 0.4 and then induced with 1 mM IPTG for 4 hr at 30°C. A soluble extract (10 ml) was prepared in buffer 1 as indicated above. Five milliliters of the extract (32 mg/ml) was loaded at a flow rate of 1 ml/min onto a 13-ml (length, 17 cm; diameter, 1 cm) anion-exchange column (diethylaminoethyl-cellulose, DE52; Whatman) equilibrated with buffer 1. The column was washed with 50 ml of buffer 1 and developed with a 50–300 mM NaCl linear gradient.

Miscellaneous. Published methods were used for immunoblotting (31), for cleavage assay (32), and for mitochondria preparation (33). Solubilization and renaturation of overproduced β subunit from mitochondrial extracts of the yeast G10 strain were carried out as described (26). Protein concentrations of the *E. coli* extracts and of the mitochondrial matrix extracts were determined with the bicinchoninic acid (BCA) protein assay reagent (Pierce) with bovine serum albumin as a standard. Quantitative immunoblotting was performed by using ^{125}I-labeled protein A. Intensities of autoradiography bands were determined using an LKB 2202 Ultroscan laser densitometer coupled to a Hewlett–Packard 3390A integrator.

RESULTS

Individual and Coexpression of the Mature α and β Subunits of the Peptidase. Anchored PCR was used to construct genes encoding the mature form of each subunit. Each gene was cloned individually into the *E. coli* pMG1 expression vector downstream the IPTG-inducible *tac* promoter. In the absence of inducer, the expression was repressed by the LacI repressor encoded by the same plasmid. Both genes were also cloned into the same expression vector as an operon. The gene encoding the mature α subunit was placed immediately downstream from the gene encoding the β subunit. As a result, both subunits should be made from a single mRNA, with the sequence between the two genes allowing reinitiation of translation. The *E. coli* strains expressing the α subunit, the β subunit, or both subunits are termed S-α, S-β, and S-αβ, respectively.

Properties of the Subunits Expressed in *E. coli*. As expected, S-α and S-β strains expressed the α subunit and the β subunit, respectively, whereas S-αβ coexpressed both subunits (see below). The subunit-specific rabbit antisera used for immunoblotting did not show any significant crossreactivity with *E. coli* proteins (data not shown). In all experiments, expression of the subunits in *E. coli* was performed at 30°C in order to minimize the appearance of degradation products. Supernatants from clarified sonicated lysates of the induced *E. coli* cells and mitochondrial matrix extracts of the VGA yeast strain (the VGA strain overproduces both subunits of the peptidase) were analyzed by immunoblotting (Fig. 1). The α and β subunits produced by the S-α and S-β strains comigrated with the α and β subunits from mitochondrial matrix, suggesting the integrity of both proteins (Fig. 1*A*). However, a major soluble degradation product of the β subunit was observed in the S-β extract. More than 50% of the subunits produced by the S-α and S-β strains were recovered in the supernatant fraction of the clarified lysates (data not shown). This result was unexpected for the β subunit, which aggregates in yeast mitochondria when not associated with the α subunit (26).

The amounts of subunits produced by the different strains are given in Table 1. The S-α produces large amounts of the α subunit, representing 20% of the total soluble proteins. The amino terminus of the overproduced α-subunit was determined [after SDS/PAGE and blotting to a poly(vinylidene difluoride) membrane] as Ala-Arg-Thr-Asp-Asn. This is the correct amino terminus of the authentic mature yeast protein

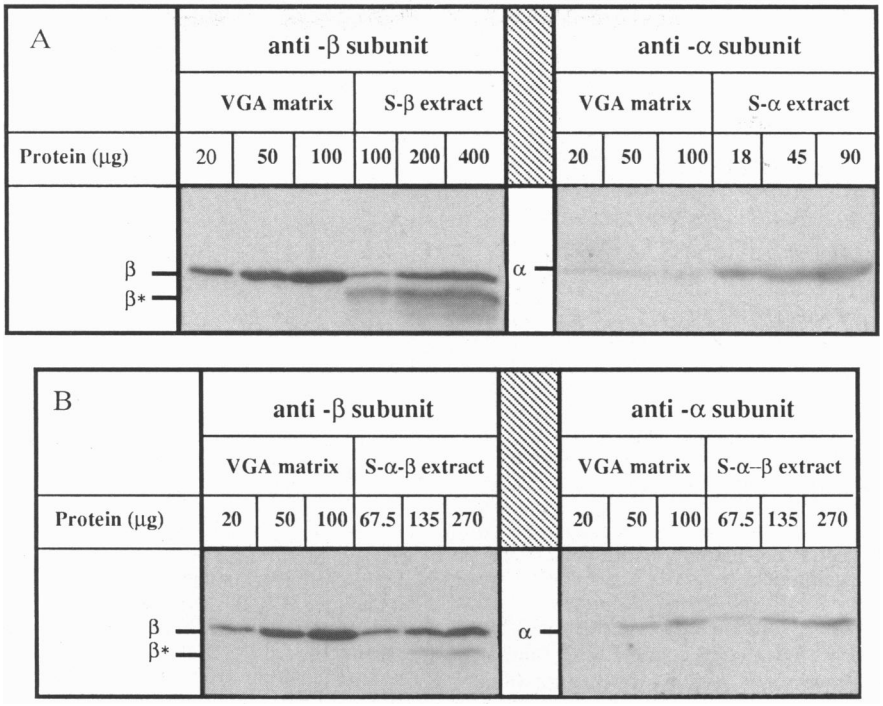

FIG. 1. (*A*) Most of the subunits are soluble in the *E. coli* cytoplasm. (*B*) Coexpression of the α and β subunits inhibits degradation of the β subunit. Soluble protein extracts from S-α, S-β, and S-αβ strains were analyzed together with matrix fraction from the peptidase-overproducing yeast strain VGA (see *Materials and Methods*) by immunoblotting for the presence of the α and β subunits. Amounts tested are indicated. Immune complexes were visualized with ^{125}I-labeled protein A. β*, degradation product of the β subunit.

(20). The amino-terminal methionine residue introduced by the gene construction was removed by *E. coli*.

Coexpression of the Subunits Inhibits Degradation of the β Subunit. Both subunits were recovered in nearly stoichiometric amounts in the S-αβ strain (Fig. 1*B* and Table 1). When expressed together in the same cell, degradation of the β subunit was very low, suggesting stabilization of the β subunit by the α subunit (Fig. 1*B*). Amounts of the holopeptidase in supernatants from S-αβ were 2- to 3-fold lower than those recovered from the mitochondrial matrix of the holopeptidase-overproducing yeast strain. However, purification from an *E. coli* lysate is much simpler and faster than purification from yeast mitochondria.

Purification of the α Subunit. The α subunit was purified from the *E. coli* S-α strain by a single chromatographic step. The purified subunit migrated as a single band of the expected molecular weight and was recognized by antiserum against the α subunit (data not shown). Milligram amounts of the α subunit were obtained with a purity of 80% (Table 2).

Cleavage Activity of the Peptidase and of Its Subunits Produced in *E. coli*. Cleavage activities of the individual subunits and of the holopeptidase expressed in *E. coli* were assayed with an artificial precursor containing the presequence of ATPase subunit 9 fused to dihydrofolate reductase (pre-Su9–DHFR). This substrate contains two characteristic cleavage sites for the matrix peptidase and is thus a good substrate for testing the specificity of cleavage (8). Coexpression of both subunits resulted in a functionally active enzyme (Fig. 2), suggesting that the two subunits assemble with each other in *E. coli*. The S-αβ extract almost completely converted the precursor and the intermediate forms to the mature form. As control, yeast mitochondrial matrix extracts processed the precursor form into intermediate and mature forms (Fig. 2, lanes 1 and 2). The efficiencies of pre-Su9–DHFR processing by the extracts from S-αβ and the VGA matrix were compared (Fig. 3*B*). When the cleavage activity was plotted versus the amounts of β subunit present in each extract, the S-αβ extract processed pre-Su9–DHFR as efficiently as the VGA matrix extract (Fig. 3*A*). Full activity of the enzyme was thus recovered from *E. coli* soluble cytoplasmic extracts. To confirm that the α subunit was not active by itself, purified α subunit and the S-α extract were assayed for cleavage activity in the presence or absence of S-β extract. Large amounts of S-α extract (80 μg, containing 20 μg of the α subunit) or of α subunit purified from the *E. coli* S-α strain (16 μg) did not show any cleavage activity (Fig. 2, lanes 6 and 7). In both cases, processing

Table 1. Amounts of peptidase and of its subunits produced by the S-α, S-β, and S-αβ strains

	% total protein				
Subunit	VGA matrix	S-α extract	S-β extract	S-αβ extract	G10 fraction
α	1.5	20	0	0.8	0
β	2	0	0.36	0.6	0.31

Amounts of soluble α and β subunits were estimated by scanning of immunoblots in which five different amounts of soluble extracts from S-α, S-β, and S-αβ were analyzed in parallel with 200, 400, 600, 800, 1000, and 1200 ng of purified α subunit and with 40, 80, 120, 160, and 200 ng of purified holopeptidase. Values are expressed as (μg of subunit/μg of total protein) × 100. The G10 fraction is a mitochondrial fraction from the β-subunit-overproducing yeast strain (see *Materials and Methods*).

Table 2. Purification of the α subunit from the α-subunit-producing *E. coli* transformant S-α

	Volume, ml	Protein, mg	α subunit, mg	Yield, %
Culture	400	ND	60	100
S-α extract	5	185	37	60
DE52 eluate	35	11	8.8	14.6

Total protein was determined by BCA assay (Pierce). Purity of the α subunit was roughly estimated from SDS/PAGE with Coomassie blue staining. ND, not determined.

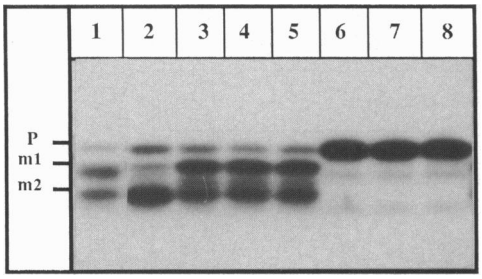

FIG. 2. Processing of pre-Su9–DHFR by the S-α, S-β, and S-$\alpha\beta$ extracts and by the purified α-subunit. Cleavage of the radiolabeled precursor pre-Su9–DHFR synthesized in a reticulocyte lysate was assayed for 30 min at 30°C in a final volume of 25 μl. The extracts were desalted prior to the assay. Samples were analyzed by SDS/PAGE and fluorography. Lane 1, VGA matrix (20 μg); lane 2, S-$\alpha\beta$ extract (40 μg); lanes 3 and 4, S-β extract (80 μg) mixed with 80 ng or 160 ng, respectively, of α subunit purified from the S-α extract; lane 5, S-β extract (80 μg) mixed with S-α extract (400 ng); lane 6, S-α extract (80 μg); lane 7, α subunit purified from the S-α extract (16 μg); lane 8, S-β extract (400 μg). P, precursor form; m1, first cleavage product; m2, second cleavage product.

activity was restored (Fig. 2, lanes 3–5) by addition of S-β extract. The S-β extract was inactive by itself (Fig. 2, lane 8).

Reconstitution of the Processing Activity from Individual Subunits. The efficiency of reconstitution is shown in Fig. 4. The specific activity of the reconstituted enzyme (50% cleavage for 100 ng of each added subunit) was lower than previous values obtained when α and β subunits purified from yeast mitochondria were mixed in stoichiometric amounts (50% cleavage for 40 ng of each subunit). However, the reconstitution of the processing activity was specific, since the same cleavage products were obtained as with the authentic holopeptidase. The reconstitution efficiency with the purified α subunit was similar to that with the S-α extract, indicating that the purification procedure did not denature the α subunit (Fig. 4). Processing activity was also reconstituted when purified α subunit or the S-α extract was mixed with a soluble mitochondrial fraction containing the β subunit free of α subunit (G10 fraction; see *Materials and Methods* and ref. 26).

DISCUSSION

The mature forms of the matrix processing peptidase subunits were expressed individually or coexpressed in *E. coli*. In both cases, subunits assembled into a functional enzyme. The processing activity was fully recovered when the two subunits were coexpressed and was significantly recovered when the subunits were expressed individually. Expressing the precursor forms of the subunits in *E. coli* did not lead to the generation of active enzyme (data not shown).

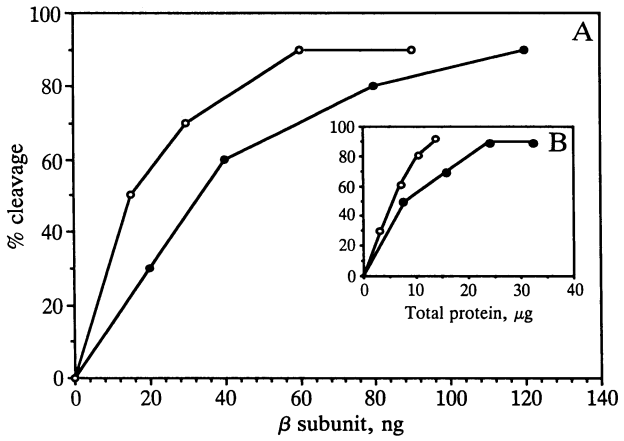

FIG. 3. S-$\alpha\beta$ extract processes pre-Su9–DHFR nearly as efficiently as VGA matrix extracts. *In vitro* synthesized pre-Su9–DHFR was incubated with increasing amounts of S-$\alpha\beta$ extract (○) or VGA matrix extract (●). Percent cleavage, which expresses the ratio of mature form to processed forms (mature plus intermediate), is plotted versus amount of β subunit (*A*) and versus total protein (*B*).

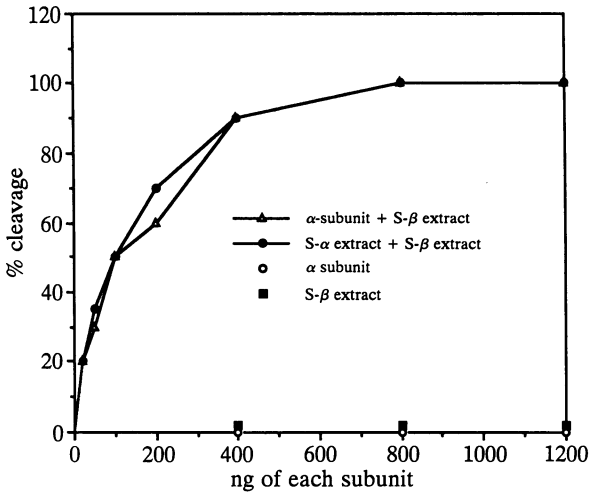

FIG. 4. The S-α extract and the α subunit purified from the S-α extract are activated by the addition of S-β extract. The S-α extract or the purified α subunit was mixed with the S-β extract in order to obtain the α and β subunits in stoichiometric amounts in the reconstitution assay. Cleavage of the substrate (pre-cytochrome oxidase subunit IV–DHFR synthesized in a reticulocyte lysate) was assayed for 45 min at 30°C in a total volume of 25 μl. Percent cleavage expresses the ratio of mature form to precursor form.

Schatz and coworkers (11) have shown that the yeast enzyme is a heterodimer composed of an α and a β subunit. The stable heterodimeric structure of the matrix processing peptidase in yeast and rat liver, but apparently not in *Neurospora* (27), is also suggested by the observation that coexpression of the α subunit stabilized the β subunit in *E. coli* cells.

The α subunit was produced in large amounts and purified. The α subunit was inactive by itself. These results confirm and extend previous data (26) indicating that the α subunit is not active by itself and should not be viewed as the "catalytic" subunit of the peptidase. Subunits from *N. crassa* were purified as monomers, and the α subunit was found to exhibit residual processing activity (10). However, it was not excluded that this activity reflected contamination of the α subunit (termed MPP) by the β subunit (termed PEP).

The availability of this expression system will facilitate further investigation of the structure and function of the peptidase and of its subunits. The expression system might be used to express processing peptidases from different sources.

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