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A purified precursor polypeptide requires a cytosolic protein fraction for import into mitochondria

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The β-subunit of mitochondrial ATPase is coded by a nuclear gene, synthesized outside the mitochondria as a larger precursor and imported into mitochondria. The β-subunit precursor was purified from yeast, both as a homogeneous, unlabeled polypeptide and in radiochemically pure form. Both precursor preparations were cleaved to the mature β-subunit by partially purified processing protease from the mitochondrial matrix. However, import of the radiochemically pure precursor into isolated yeast mitochondria required a cytosolic fraction from yeast or reticulocytes. The cytosolic factor was non-dialyzable and trypsin-sensitive; its apparent mol. wt. was ~40 000 as judged by gel filtration. Import of some proteins into mitochondria thus requires proteins of the 'soluble' cytoplasm.

Key words: mitochondrial ATPase/mitochondria/protein imports/cytosolic protein

Introduction

Most of the several hundred mitochondrial proteins are coded by nuclear genes, synthesized outside the mitochondria, and imported into mitochondria. So far, the mechanism of this import process has been studied by pulse-chase experiments with intact cells and by incubating isolated mitochondria with radiolabeled mitochondrial precursor polypeptides that had been synthesized in a cell-free protein synthesizing system (Schatz and Butow, 1983; Hay *et al.*, 1984). These experiments have revealed the existence of several distinct import routes. Import into the matrix or the inner membrane usually involves the following steps: (i) synthesis of a precursor polypeptide with an amino-terminal extension on free cytoplasmic polysomes; (ii) release of the precursor into a cytosolic pool; (iii) binding of the precursor to a receptor-like component on the mitochondrial surface; (iv) energy-dependent translocation across both membranes; and (v) removal of the amino-terminal extension by a chelator-sensitive protease in the mitochondrial matrix.

To understand the molecular events underlying this pathway, the individual components must be obtained in a homogeneous and functional state. This is particularly difficult for the larger precursor polypeptides since, *in vivo*, their life-times are only of the order of minutes and their steady-state concentrations are usually very low (Reid and Schatz, 1982b). Here we report the isolation of 100-μg amounts of homogeneous precursor to the F_1-ATPase β-subunit. We also show that import of the isolated precursor by energized yeast mitochondria requires a 40-kd protein fraction from the yeast cytoplasm.

Results

Isolation of homogeneous β-subunit precursor

Growth of a *rho*$^-$ yeast mutant in the presence of carbonylcyanide-*m*-chlorophenyl hydrazone (CCCP) leads to a dramatic accumulation of mitochondrial precursor polypeptides (Reid and Schatz, 1982a). Even then, however, the intracellular concentration of these precursors is quite low, dictating purification factors of ~10 000. We chose to purify the precursor to the ATPase β-subunit mainly because experiments with intact *rho*$^-$ cells have shown that it is relatively stable and that it retains the capacity to be imported into mitochondria for considerably longer than most other mitochondrial precursor polypeptides (Reid and Schatz, 1982a). The key steps of the isolation procedure are: (i) rapid denaturation of the yeast cellular proteins to inactive proteases; (ii) extraction with SDS; (iii) immune adsorption of mature β-subunit and its precursor; (iv) separation of the precursor from the mature subunit by preparative isoelectric focussing; and (v) renaturation of the denatured precursor. 85 g of wet-weight cells yielded ~100 μg of pure precursor (Table I and Figure 1A). If analyzed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Blue, the final product was essentially homogeneous (Figure 1A). The faint band migrating more slowly than the precursor is a chemically modified form of the precursor polypeptide as its relative amount increases by prolonged treatment of the precursor with Triton X-100 (not shown). Similarly, silver staining failed to reveal contaminating bands exceeding 1% of the precursor band (not shown).

When a scaled-down version of this purification procedure was applied to *rho*$^-$ cells that had been labeled with $^{35}SO_4^{2-}$ in the presence of CCCP, radiochemically pure β-subunit precursor was obtained (Figure 1B). Owing to the problems of working with pulse-labeled cells, this labeled precursor was chemically only ~30% pure (Figure 1C). Subsequently, a simpler procedure was adopted (Materials and methods) that yielded radiochemically pure β-subunit containing unlabeled

Table I. Purification of β-subunit precursor

Purification Step	Volume (ml)	Protein (mg)	β-subunit precursor (μg)[a]	Yield (%)	Purification (fold)
Extraction with SDS	300	6300	620	(100)	–
Immune column	7.8	1.38	270	44	2030
Chromatofocussing	4.8	0.58	150	24	2870
Isoelectric focussing	1.5	n.d.	110	18	~10 000[b]

n.d. = not determined

[a]Determined by immunoblotting. The method was standardized with authentic F_1-ATPase by assuming that the rabbit antiserum used reacted equally well with the SDS-treated mature and precursor form of the β-subunit.

[b]Inferred from the facts that the final precursor preparation was free from impurities (Figure 1A) and that the precursor accounted for 0.01% of the protein in the initial SDS extract(see first horizontal column of this Table).

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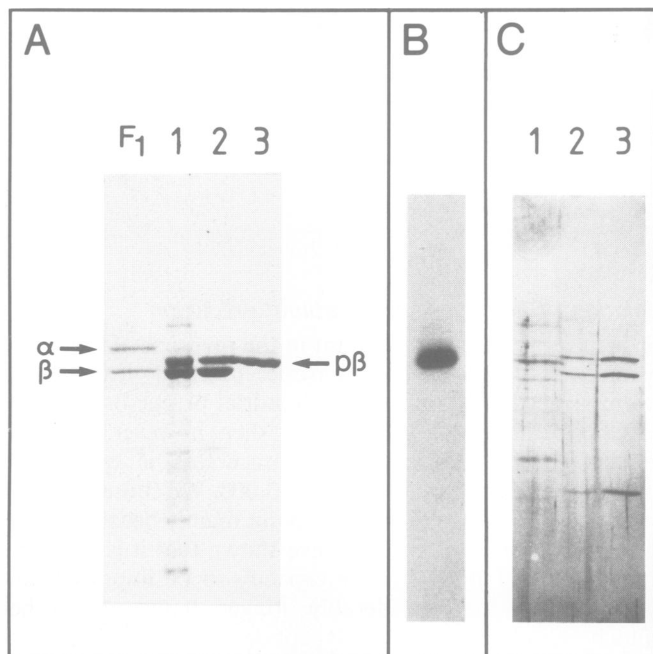

Fig. 1. Isolation of chemically and radiochemically pure precursor to the F_1-ATPase β-subunit. The precursor was isolated from unlabeled **(A)** or labeled **(B,C)** yeast cells (see Materials and methods). Aliquots of the precursor at different stages of purification were analyzed by SDS-polyacrylamide gel electrophoresis. **(A)** Gel was stained with Coomassie Brilliant Blue R. **F_1**, F_1 standard (0.3 μg); **1,** eluate from immune column (2.7 μg); **2,** eluate from chromatofocussing column (2.0 μg); **3,** after isoelectric focussing (1.1 μg). **(B)** Gel was dried and fluorographed. Purified radiolabeled precursor (3200 c.p.m.) was analyzed. **(C)** Gel was stained with silver nitrate. **1,** purified radiolabeled precursor (3200 c.p.m.); **2,** F_1 standard (3 ng); **3,** F_1 standard (12 ng). The positions of the mature α- and β-subunit and of the β-subunit precursor (pβ) are indicated by arrows.

mature β-subunit and bovine serum albumin (BSA) as a carrier. Import experiments with isolated mitochondria failed to reveal any difference between the labeled precursors isolated by the two procedures.

The homogeneous unlabeled precursor and the radiochemically pure precursor can be processed to mature β-subunit

Upon renaturation at pH 7.4, more than two-thirds of the homogeneous, unlabeled precursor could be processed to the mature form by a partially-purified preparation of chelator-sensitive protease from the yeast mitochondrial matrix (Figure 2). The same result was obtained with the radiochemically pure precursor preparation (Figure 3). As expected (Böhni *et al.*, 1983), processing was sensitive to 1,10-phenanthroline (Figures 2, 3) but insensitive to inhibitors of mitochondrial energy metabolism (Figure 3). No processing was observed if the precursor was renatured at pH 6.0 and then exposed to the protease at pH 7.4 (not shown). This confirms the observation (Böhni *et al.*, 1983) that the matrix protease fails to cleave denatured precursors. The results of Figures 2 and 3 indicate, therefore, that at least two-thirds of the precursor molecules had been renatured.

Import of isolated precursor into mitochondria requires a cytosolic factor

The radiochemically pure β-subunit precursor was only very inefficiently processed by energized yeast mitochondria (Figure 4, lane 2); processing was stimulated by unlabeled reticulocyte lysate (Figure 4A) or a cytosolic fraction from

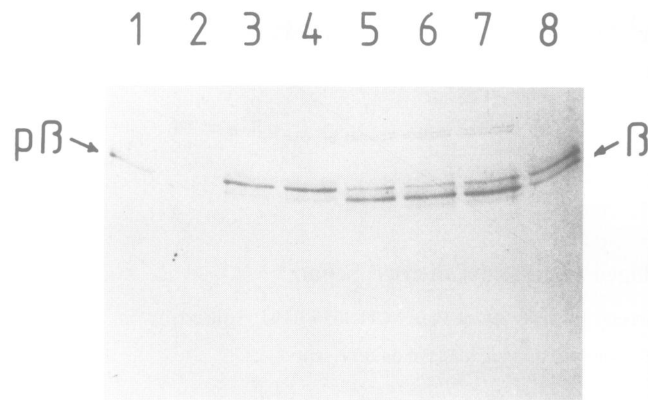

Fig. 2. Homogeneous unlabeled β-subunit precursor is cleaved to the mature form by mitochondrial matrix protease. Pure unlabeled β-subunit precursor (100 ng) was renatured at pH 7.4 (Materials and methods) and incubated at 28°C in 200 μl with matrix protease (purified through step 2, Böhni *et al.*, 1983) in 20 mM Hepes-KOH pH 7.5, 75 mM KCl, 50 μM $CoCl_2$, 50 μM $ZnCl_2$, 1 mM DTT and 0.6 M sorbitol as described below. The samples were dissociated with SDS at 95°C and analyzed by SDS-polyacrylamide gel electrophoresis followed by immune blotting with rabbit antiserum against the β-subunit. To remove most of the mature β-subunit contaminating the matrix protease, 400 μl of the enzyme solution was shaken for 1 h with 100 μl of Sepharose 4B beads containing covalently-bound antibody against the β-subunit (the same type of column as used for isolating the β-subunit precursor). **1,** precursor alone; **2,** 10 units of matrix protease (note the slight residual contamination by mature β-subunit); **3,** precursor + 10 units of matrix protease, no incubation; **4,** precursor + 10 units of matrix protease + 2 mM 1,10-phenanthroline, 45 min incubation; **5,** precursor + 10 units of matrix protease, 5 min incubation; **6,** same as **5,** but 15 min incubation; **7,** same as **5,** but 45 min incubation; **8,** same as **7,** but 1 unit of matrix protease.

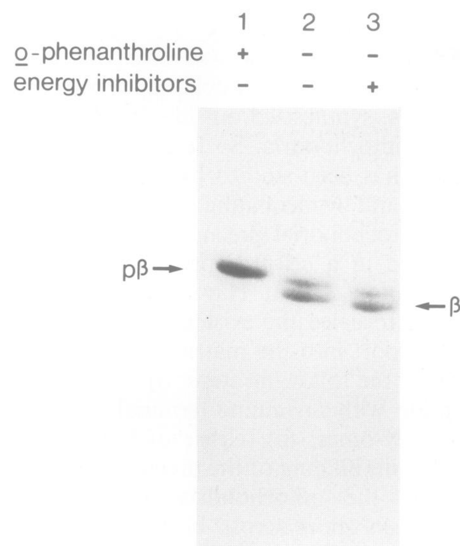

Fig. 3. Radiochemically pure renatured β-subunit precursor is cleaved to the mature form by mitochondrial matrix protease. Aliquots of radiolabeled, renatured β-subunit precursor (5000 c.p.m. ^{35}S) were incubated with 10 units of yeast mitochondrial matrix protease as described in Figure 2 for 30 min at 27°C, dissociated with SDS at 95°C and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. **1,** with 2 mM 1,10-phenanthroline; **2,** no inhibitors; **3,** with 2 mM KCN, 6 μg oligomycin and 10 μg atractyloside. The arrows indicate the position of the mature β-subunit (β) and the precursor of the β-subunit (pβ).

yeast (Figure 4B). The stimulation varied between experiments, but was usually between 4- and 8-fold (Figure 4, bottom). Stimulation of processing by isolated mitochondria was sensitive to inhibitors of mitochondrial energy metab-

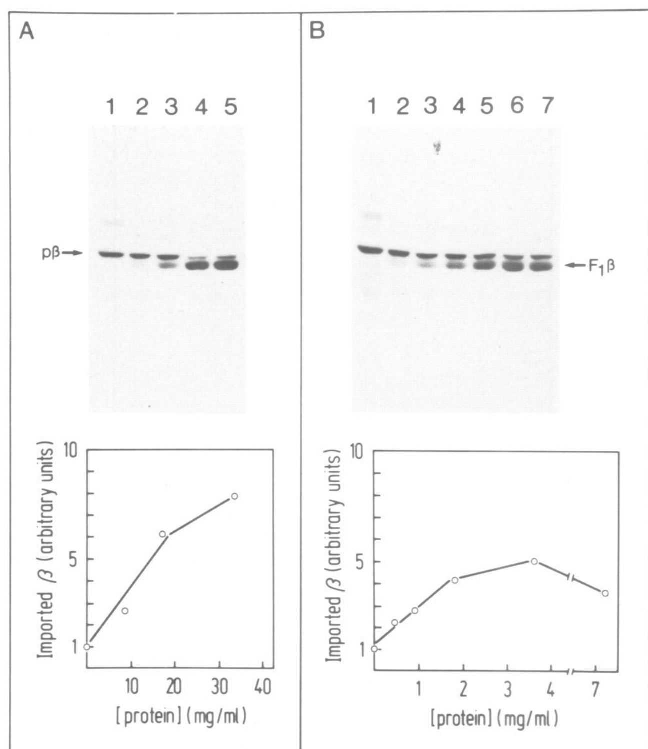

Fig. 4. Processing of radiochemically pure β-subunit precursor by yeast mitochondria requires a cytosolic component from reticulocytes or from yeast. Purified ^{35}S-labeled β-subunit precursor (10 290 c.p.m.) was incubated for 30 min at 27°C in a final volume of 400 μl, with 250 μg of yeast mitochondria and the indicated amounts of cytosolic fractions in the presence of 20 mM Hepes-KOH pH 7.4, 0.6 M sorbitol, 1 mM ATP, 5 mM GTP, 5 mM phosphoenolpyruvate, 4 units of pyruvate kinase, 1 mM $MgCl_2$ and 75 mM KCl. The mitochondria were then re-isolated by centrifugation through a 1 ml cushion of 0.6 M sucrose, 20 mM Hepes-KOH pH 7.4, 2 mM EDTA, 2 mM 1,10-phenanthroline, 1 mM PMSF, 0.1 mM TLCK, dissociated in 2% SDS at 95°C and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. **(A) 1,** pre β-subunit alone (1029 c.p.m.; 10% of sample present in each incubation); **2,** after incubation with mitochondria; **3–5,** after incubation with mitochondria and 8.4, 16.8 and 33.6 mg/ml reticulocyte lysate, respectively. **(B) 1,** pre β-subunit alone (1029 c.p.m.; 10% of total sample); **2,** after incubation with mitochondria; **3–7,** after incubation with mitochondria and 0.45, 0.9, 1.8, 3.6 and 7.2 mg/ml yeast (pep-4) cytosolic fraction. The labeled bands were quantified by scanning the exposed film and stimulation was calculated on the basis of mature radiolabeled β-subunit generated (lower panels). The arrows indicate the positions of the mature β-subunit ($F_1\beta$) and the precursor (pβ), respectively.

olism (Figure 5) and yielded labeled mature β-subunit that was resistant to externally-added trypsin (Figure 6). The stimulatory effect of cytosolic extracts on processing by isolated mitochondria thus reflects stimulation of import of the precursor into mitochondria.

We conclude that import of the β-subunit precursor into yeast mitochondria requires one or more components present in the cytosol of reticulocytes or of yeast.

Properties of the cytosolic yeast factor

The stimulatory agent(s) present in the cytosolic reticulocyte and yeast fractions can be precipitated by ammonium sulfate (Materials and methods) and inactivated by trypsin (Figure 7), suggesting the involvement of protein(s). Upon gel filtration on Sepharose 6B (Figure 8), the stimulatory activity present in the yeast cytosol fraction migrates with a mol. wt. of ~40 000 and is separated from the bulk of cytosolic proteins. To quantify the data of Figure 8, different amounts of

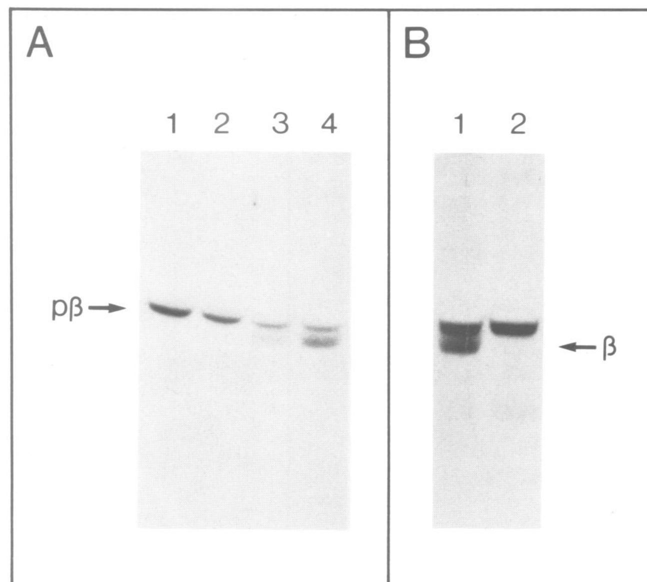

Fig. 5. Processing of radiochemically pure β-subunit precursor by mitochondria plus cytosolic fraction is energy-dependent (i.e. reflects import of the precursor into mitochondria). The experiment was carried out essentially as described in Figure 3 with the following exceptions: where indicated, reticulocyte lysate (21.5 mg/ml), yeast (*pep-4*) cytosolic fraction (2.9 mg/ml) or energy poisons (2 mM KCN, 25 μg atractyloside, 8.4 μg oligomycin) were added to the incubation with mitochondria. The energy poisons (1 mg/ml atractyloside, 0.336 mg/ml oligomycin and 3 mM KCN) were pre-incubated with the mitochondria (10 mg/ml) for 10 min at 0°C before addition of precursor and cytosolic fractions, and KCN was added to the assay mixture to 2 mM. **(A) 1,** plus energy poisons; **2,** plus energy poisons plus reticulocyte lysate; **3,** no additions; **4,** plus reticulocyte lysate. **(B) 1,** plus yeast cytosol; **2,** plus yeast plus energy poisons. pβ = β-subunit precursor; β = mature β-subunit.

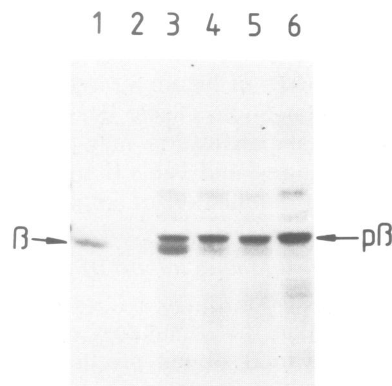

Fig. 6. Processing of radiochemically pure β-subunit precursor by mitochondria plus cytosolic fraction yields mature β-subunit that is resistant to externally-added trypsin. Purified [^{35}S]β-subunit precursor (15 000 c.p.m.) was incubated for 30 min at 27°C with 250 μg of yeast mitochondria and the indicated amounts of unlabeled reticulocyte lysate as described in Figure 4. The samples were then incubated at 0°C for 20 min with or without 125 μg/ml trypsin. All samples were mixed with 1 mM PMSF and 2.5 mg/ml trypsin inhibitor, sedimented through a sucrose cushion and analyzed as in Figure 4. **Lanes: 1,** plus 36 mg lysate/ml, plus trypsin; **2,** no lysate, plus trypsin; **3,** plus 36 mg lysate/ml, no trypsin; **4,** plus 18 mg lysate/ml, no trypsin; **5,** plus 9 mg lysate/ml, no trypsin; **6,** no lysate, no trypsin. pβ = β-subunit precursor; β = mature β-subunit.

each fraction were assayed for stimulating import of radiolabeled β-subunit precursor into yeast mitochondria; values from the linear part of the dose-response curve were used to calculate units of activity. One unit was defined as the

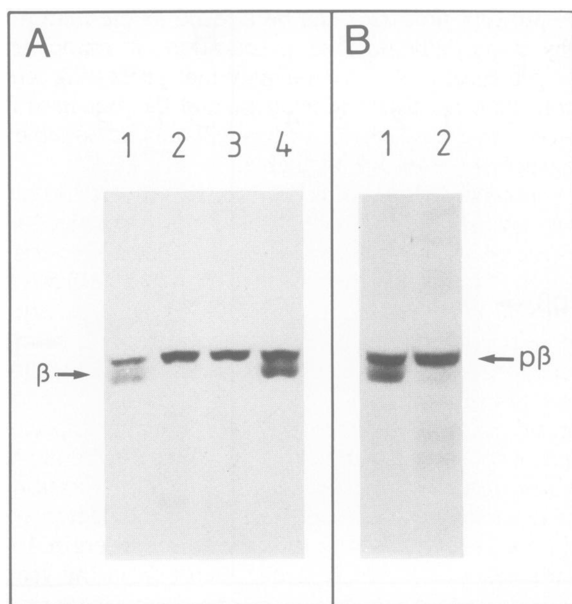

Fig. 7. The cytosolic factor is trypsin-sensitive. The cytosolic fractions were treated with trypsin or a mixture of trypsin and trypsin inhibitor as described below. Import of the radiochemically pure β-subunit precursor into isolated yeast mitochondria was then assayed as described in Figure 4. **(A) 1,** untreated reticulocyte lysate (10.32 mg); **2,** no further additions; **3,** reticulocyte lysate (10.32 mg) that had first been incubated with 4 μg trypsin (15 min; 27°C) and then with 80 μg trypsin inhibitor (5 min; 27°C); **4,** same as **3,** except that the first incubation was without additions and the second incubation with both trypsin and trypsin inhibitor. **(B) 1,** untreated cytosolic fraction from yeast (1.16 mg; *pep-4* mutant); **2,** same as **1** except that the yeast cytosolic fraction had first been incubated with 5 μg trypsin (10 min; 27°C) and then with 50 μg trypsin inhibitor (5 min; 27°C). pβ = β-subunit precursor; β = mature β-subunit.

amount of factor which stimulated precursor import into mitochondria 2-fold over the factor-free control. Evaluation of the experiment shown in Figure 8 revealed that gel filtration purifies the factor 6-fold (Table II); since recovery was only 36%, the actual purification may have been higher. While the data of Figure 8 and Table II are preliminary, they strongly suggest that stimulation of import is caused by a specific component present in the yeast cytosol.

Cytosolic factor also stimulates import of precursor polypeptides synthesized in vitro

It could be argued that the stimulatory effects of cytosolic fractions are limited to β-subunit precursor that had been purified and then renatured *in vitro*. Figure 9 shows that this is unlikely: the precursor of yeast cytochrome b_2 was synthesized in a reticulocyte lysate in the presence of yeast mRNA and [^{35}S]methionine and the labeled lysate was then passed through a Sephadex G-25 column. When low concentrations of this labeled lysate were incubated with energized mitochondria, very little of the radiolabeled cytochrome b_2 was processed to the mature form. (As discussed by Daum *et al.*, 1982b, conversion of the cytochrome b_2 precursor to the mature form is a reliable test for import into mitochondria.) Addition of unlabeled reticulocyte lysate (as a source of cytosolic factor) stimulated import of the *in vitro* synthesized cytochrome b_2 precursor 4-fold.

Discussion

Proteins destined to be imported into mitochondria are synthesized as precursors whose conformations appear to differ from those of the corresponding mature polypeptides (Schatz and Butow, 1983). Most precursors also carry an amino-terminal extension whose precise role in the import process is not clear; does the extension recognize a mitochondrial surface receptor or does it merely stabilize the conformation of the precursor molecule? How does the conformation of a precursor polypeptide differ from that of the corresponding mature polypeptide? How quickly does the precursor chain refold upon removal of the amino-terminal extension? These questions can only be studied rigorously with amounts of precursor sufficient for chemical and physico-chemical experiments.

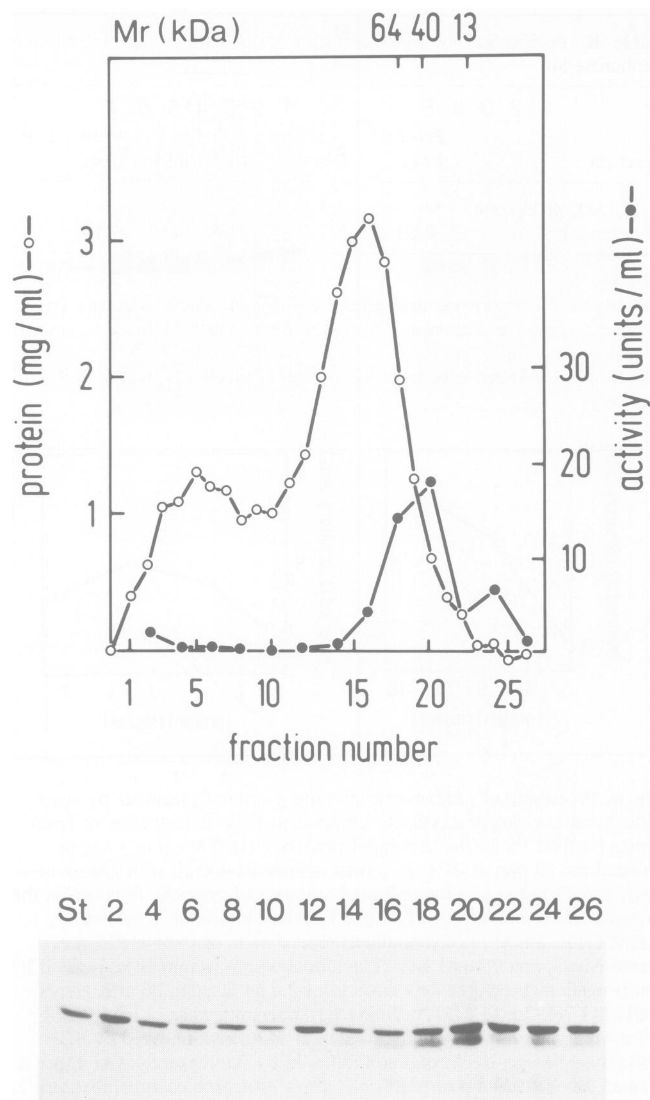

Fig. 8. The yeast factor can be separated from the bulk of cytosolic proteins. A cytosolic fraction (1.5 ml ammonium sulfate fraction, see Materials and methods; 54 mg protein) of the *pep-4* yeast mutant was passed through a Sepharose 6B column (40 ml bed volume; 1 x 50 cm) which had been equilibrated with 20 mM Hepes-KOH pH 7.4, 0.1 M KCl. The column was developed with the same buffer. Fractions of 1 ml were collected and assayed for protein **(upper panel)** as well as for their stimulatory effect on import of β-subunit precursor into mitochondria **(lower panel)**; see Figure 4 for details; 150 μl from each fraction was used for the import assay). The radioactive bands shown in the lower panel were quantified by densitometry and the results were plotted as 'units/ml' in the upper panel. Fraction numbers are counted starting from the void volume. Blue dextran, rabbit hemoglobin, bovine cytochrome *c* and K_3 [Fe(CN)$_6$] were used as mol. wt. standards. St indicates the precursor of the $F_1\beta$-subunit.

Table II. Purification of the yeast cytosolic factor by gel filtration on Sepharose 6B

Fraction	Protein (mg)	Activity (units)	Specific Activity (units/mg)	Purification (fold)
$(NH_4)_2SO_4$ precipitate	54	255	4.7	–
Fraction 20[a]	0.65	17.9	27.5	5.9

[a]See Fig. 8.
Stimulation of import was measured with different amounts of each fraction and activities were determined from the linear parts of the dose-response plots.
The total activity recovered from the Sepharose 6B column was 93 units (36% recovery).

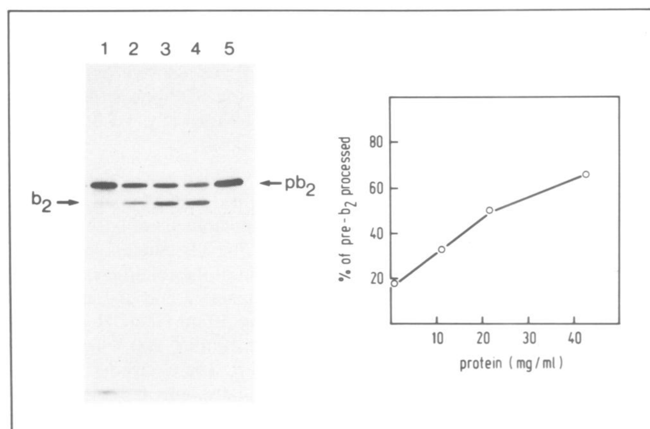

Fig. 9. Reticulocyte lysate stimulates import of pre-cytochrome b_2 into yeast mitochondria. A reticulocyte lysate was programmed with yeast RNA and labeled with [^{35}S]methionine (Maccecchini *et al.*, 1979). The translated lysate (0.1 ml; 4 x 10^5 acid-insoluble c.p.m./μl) was passed through a 0.8 x 4 cm column of Sephadex G-25 (fine) which had been equilibrated with 20 mM Hepes-KOH, 0.1 M KCl. Aliquots (270 μg of protein) of the filtered, labeled lysate were used as a source of labeled pre-cytochrome b_2 in the standard import assay (Gasser *et al.*, 1982) except that import was tested for 15 min at 27°C with 80 μg of mitochondria in the presence of varying amounts of unfiltered, unlabeled reticulocyte lysate. The mixture was directly dissociated in SDS and processing of radiolabeled cytochrome b_2 precursor was analyzed as usual (Gasser *et al.*, 1982). **Lanes 1–4**: 0.27 mg labeled lysate incubated with mitochondria in the presence of 0, 4.2, 8.4 and 16.8 mg unlabeled reticulocyte lysdate; **5**: 0.27 mg labeled lysate alone. The radioactive bands shown in the left panel were quantified by densitometry of the exposed X-ray film and the data were plotted in the right panel. Since the calculation of '% processing' assumes that no radioactivity is lost from the polypeptide, the values are underestimations. b_2 and pb_2 indicate cytochrome b_2 and pre-cytochrome b_2, respectively.

Here we report, for the first time, the isolation of a pure precursor polypeptide carrying a cleavable amino-terminal extension. The preparation is essentially homogeneous and at least two-thirds of the molecules are 'native' in that they can be processed by mitochondrial matrix protease.

The study of this precursor should be facilitated by the recent isolation of the corresponding gene from yeast (Saltzgaber-Müller *et al.*, 1983). For example, elegant gene fusion studies have already shown that the amino acid sequences targeting the precursor to mitochondria are located in the amino-terminal half of the molecule (Douglas *et al.*, 1983). Also, milligram amounts of the homogeneous precursor should soon be available from yeast strains overproducing this precursor (Ohta and Reid, in preparation).

The purified precursor can be cleaved to the mature subunit by a partially purified preparation of mitochondrial matrix protease; it is, thus, unlikely that processing requires components other than the protease and the precursor itself. However, conclusive proof for this will only be possible once homogeneous protease is available.

In a convincing series of experiments, Neupert and his colleagues have shown that cytochrome *c* is synthesized as the heme-free apoprotein which lacks a cleavable amino-terminal extension. As a consequence, removal of heme from holocytochrome *c* by chemical means yields apocytochrome *c* which behaves in many ways like 'cytochrome *c* precursor' produced *in vivo* or in cell-free protein-synthesizing systems (Zimmerman *et al.*, 1979). It will be interesting to compare the refolding of apocytochrome *c* (presumably triggered by attachment of heme) to any refolding of the β-subunit precursor which might be triggered by proteolytic maturation.

The most striking result of this study is the demonstration that import of the purified precursor into energized mitochondria requires a proteinaceous factor from the yeast (or reticulocyte) cytosol. A similar finding was recently reported for the import of ornithine transcarbamylase into mammalian mitochondria (Argan *et al.*, 1983; Miura *et al.*, 1983). In these experiments, the precursor molecule to be imported was synthesized in a reticulocyte lysate in the presence of [^{35}S]methionine and the proteinaceous factor of the lysate was then made limiting by dilution or by adsorption to Sephadex G-25 beads. Our own data fully confirm these earlier reports. In addition, we now show that the factor can be purified and, thus, probably represents a specific component. The demonstration that the factor is present in yeast allows its assay in a homologous system and should facilitate its isolation. Finally, we describe a defined assay system consisting only of purified precursor and energized mitochondria; this assay permits quantitation of the factor, a prerequisite for its purification. As shown in Table II, some modest purification has already been achieved.

While the factor appears to be a protein by several standard criteria, it could still be a small molecule tightly bound to (and perhaps stabilized by) proteins which are themselves inactive. For example, stimulation of mitochondrial protein synthesis by cytosolic 'proteins' is almost entirely caused by protein-bound guanyl nucleotides (Ohashi and Schatz, 1980). Until the factor described here is purified to homogeneity, its nature, its mechanism and action and, indeed, its biological relevance will remain uncertain.

Little is known about how this factor stimulates import. It probably does not act like the 'signal recognition particle' for two reasons. Signal recognition particle from eukaryotes (and its putative counterpart from bacteria) consists of a small RNA and six polypeptides (Walter and Blobel, 1982) whereas the cytosolic factor described here appears to be much smaller. Furthermore, eukaryotic signal recognition particle prevents completion of precursor polypeptide chains in the absence of the target membrane; in contrast, mitochondrial precursor polypeptides are usually imported post-translationally as completed molecules. Our experiments do not rigorously exclude the possibility that the factor merely stabilizes the electrochemical potential across the mitochondrial inner membrane. We consider this unlikely, however, because yeast mitochondria isolated by our procedure are well coupled (e.g., Gasser *et al.*, 1982) and because protein import into mitochondria only requires a rather low potential

(Reid and Schatz, 1982a). One reasonable hypothesis would be that the cytosolic factor binds to the precursors and thereby triggers their correct association with the appropriate mitochondria surface receptor(s).

Materials and methods

Yeast strains

The wild-type *Saccharomyces cerevisiae* strain D273-10B (α; ATCC 25657) and the protease-deficient *S. cerevisiae* mutant *pep-4* (Jones, 1977) were used. For preparation of mitochondria and cytosolic fractions, the cells were grown on L-lactate (Daum *et al.*, 1982a). For accumulation of mitochondrial precursors, the rho^- mutant was grown as in Reid and Schatz (1982a).

Preparative procedures

Mitochondria were prepared as in Daum *et al.* (1982a), except that the isolation medium was buffered with 20 mM Hepes-KOH pH 7.4 and that the mitochondria were washed four times.

Monoclonal antibody against mature yeast F_1-ATPase β-subunit was prepared essentially as described (Stähli *et al.*, 1980); the mice were immunized first with 50 μg of β-subunit in the presence of Freund's complete adjuvant and 3, 5 and 8 weeks later, with 10 μg of β-subunit plus Freund's incomplete adjuvant. Fusion of the spleen cells with myeloma cells (line PA I; kindly provided by Dr. H. Trachsel) was performed 4 days later. Testing of the hybridomas, subcloning by limiting dilution and preparation of ascites fluid were performed by standard procedures (McKearn, 1980). The monoclonal antibody was identified as IgG_1 by testing hybridoma culture fluid with isotype-specific antisera by Ouchterlony double-diffusion. The immunoglobulin fraction was isolated from ascitic fluids by ammonium sulfate fractionation followed by DEAE-cellulose chromatography.

Cytosolic extract of yeast was prepared from the yeast *pep-4* mutant. The cells were grown to a density of 100 Klett units, converted to spheroplasts, and disrupted (Daum *et al.*, 1982a) in 0.6 M sorbitol, 20 mM Hepes-KOH pH 7.4, 1 mM phenylmethylsulphonyl fluoride (PMSF). All subsequent steps were performed at 0–4°C. The disrupted mixture was then adjusted to 0.1 M KCl with 2.5 KCl, incubated for 10 min at 0°C and spun for 5 min at 3000 r.p.m. (Sorvall SS-34 rotor). The supernatant was freed from particles (1 h at 35 000 r.p.m.; 10^5 *g* max) and precipitated with ammonium sulfate (75% saturation); the pH was kept at 7–7.5 by adding 2 M Tris base. Precipitated protein was collected (20 min; 12 000 r.p.m.; SS-34 rotor), suspended in a minimal volume of 20 mM Hepes-KOH pH 7.4, 1 mM PMSF, 0.1 M KCl and dialyzed against the same buffer for 6 h. Aliquots were quick-frozen in liquid N_2 and stored at –70°C.

Purification of unlabeled β-subunit precursor

Step 1. Accumulation of precursor in vivo. The rho^- yeast mutant D273-10B-1 was grown at 25°C in 25 l of 2% peptone, 1% yeast extract, 0.1 M KP_i pH 6.0 and 5% glucose. When the culture had reached a density of 50 Klett units (~1.9 x 10^7 cells/ml), CCCP was added to 20 μM and growth was allowed to continue until the culture had reached 120 Klett units (usually 6 h). The cells were harvested by centrifugation (10 min at 5000 r.p.m.,-Sorvall GS-3 rotor). The yield was ~85 g (wet weight) of cells.

Step 2. Extraction of cell protein. The cells were suspended with 90 ml of H_2O, rapidly mixed with 10 ml of 100% (w/v) trichloroacetic acid (TCA) and centrifuged for 5 min at 5000 r.p.m. (Sorvall GS-3 rotor). The pellet was suspended in 200 ml of H_2O. Aliquots of 45 ml were shaken with 50 g of glass beads (0.5 mm diameter) for 1 min, at room temperature at 4000 oscillations/min in a Braun shaker (Braun Co., Melsungen, FRG). The suspension was decanted from the glass beads and the beads were used for shaking the next aliquot. When all aliquots had been processed, the beads were rinsed three times with 5% TCA. The processed aliquots and the rinses were combined and centrifuged as above. The pellet was washed twice with 1 l of ice-cold H_2O, suspended in 100 ml of 5% SDS, 1% 2-mercaptoethanol, 1 mM PMSF, 1 mM EDTA, neutralized with 2 M Tris base, shaken gently for 10 h at 30°C and centrifuged for 20 min at 12 000 r.p.m. (Sorvall SS-34 rotor). Extraction with the SDS-containing buffer was repeated twice and the extracts were combined (total volume 300 ml).

Step 3. Removal of excess SDS. The combined extracts were dialyzed for 12 h at room temperature against 5 x 5 l of H_2O, adjusted to 10 mM Tris-Cl pH 7.55, 0.1 M NaCl, 0.1 mM EDTA, 0.05% 2-mercaptoethanol, and incubated for 12 h at 0°C. Precipitated SDS was removed by centrifugation (20 min; 12 000 r.p.m.; Sorvall SS-34 rotor) and the supernatant was adjusted to 1% Triton X-100, 1 mM PMSF.

Step 4. Immune chromatography. CNBr-activated Sepharose 4B (7 g: Pharmacia) was incubated with gentle shaking overnight at +4°C, with 40 mg of the monoclonal antibody against yeast F_1-ATPase β-subunit, in 0.1 M $NaHCO_3$ and 0.5 M NaCl. The mixture was transferred to a siliconized column (1 x 25 cm). The column (bed volume = 20 ml) was washed with 1 M ethanolamine pH 8.0, then with 0.1 M Na-citrate (pH 4.0), 0.5 M NaCl and finally with phosphate-buffered saline (PBS) containing 0.01% NaN_3. The dialyzed extract obtained in Step 3 was passed through the column at +4°C at a flow rate of 20 ml/h and the column was washed successively with 30 ml of 50 mM Tris-Cl pH 8.0, 0.15 M NaCl, 1 mM EDTA, 1% Triton-X-100, 0.05% 2-mercaptoethanol and with 60 ml of 20 mM Tris-Cl pH 7.55, 0.5 M NaCl, 0.1 mM EDTA. Bound antigen was then eluted with 20 ml of 6 M urea, 25 mM imidazole Cl pH 6.4, 100 mM NH_4Cl, 1 mM dithiothreitol (DTT) and 1.6 ml fractions were collected. Aliquots of each fraction were analyzed by SDS-polyacrylamide gel electrophoresis and fractions containing mature β-subunit and its precursor were pooled (total volume = 7.8 ml). The pooled sample was dialyzed for 3 h at +4°C against 6 M urea, 25 mM imidazole Cl (pH 6.4) and 1 mM DTT.

Step 5. Chromatofocussing. A 0.5 x 30 cm column of gel PBE 94 (6 ml bed volume; Pharmacia) was washed with 25 mM imidazole Cl pH 6.5, 1 mM DTT, 6 M urea until the pH of the effluent was 6.5. The column was then loaded with the pooled sample obtained in Step 4 and developed with 100 ml of 'poly-buffer 74'-HCl (16-fold diluted; pH 4.0, Pharmacia), 1 mM DTT, 6 M urea. Fractions of 1.7 ml each were collected and assayed for mature F_1-ATPase β-subunit and the β-subunit precursor by SDS-polyacrylamide gel electrophoresis. The two forms of the β-subunit eluted at pH 5.3. Fractions containing the two forms of the subunit were pooled.

Step 6. Preparative isoelectric focussing. A 1.1 x 20 cm glass column (closed at the bottom by a flat dialysis membrane) was filled with 15 ml of a 0–20% sucrose gradient containing 8 M urea, 2% 2-mercaptoethanol, 2% Nonidet P40 (BDH Chemicals, Ltd.), 2% Ampholine 5-7 (LKB, Stockholm) and the pooled fraction obtained in Step 4. The lower end of the column was immersed in 0.2% phosphoric acid, 60% sucrose (anode) and the upper end penetrated into a perforated beaker containing 10 mM NaOH (cathode). Focussing was achieved by applying 100 V for 10 min, 200 V for 15 min, 300 V for 2 h, 400 V for 4 h and 600 V for 6 h. The electrodes were then removed and the sucrose gradient was fractionated into 0.5 ml fractions. These were again analyzed by SDS-polyacrylamide gel electrophoresis. A good separation between the mature β-subunit (pI = 5.4) and its precursor form (pI = 6.0) was obtained.

Step 7. Renaturation of the purified precursor. The precursor fraction was diluted with 2 volumes of 6 M urea, 25 mM imidazole Cl (pH 7.0) and 1 mM DTT, and loaded on PBE 94 equilibrated with 6 M urea, imidazole Cl (pH 7.0) and 1 mM DTT. The column was developed as described in Step 5. For renaturation, 300 μl of the precursor solution was neutralized with 2 M Tris and dialyzed against 10% glycerol, 20 mM Hepes-KOH (pH 7.4), 0.1 M KCl and 1 mM $MgCl_2$ for 90 min and then against 20 mM Hepes-KOH (pH 7.4), 0.1 M KCl and 0.6 M sorbitol for 90 min.

Purification of radioactive β-subunit precursor

The rho^- yeast mutant was grown overnight at 30°C to a density of 100–200 Klett units in 50 ml of 1% yeast extract, 2% peptone, 2% galactose. The cells were isolated by centrifugation, washed once with water, suspended to a density of 25 Klett units in 100 ml of synthetic medium (Wickerham, 1946), supplemented with 0.05% glucose, 2% galactose, 0.1 mM $(NH_4)_2SO_4$ and grown at 30°C to a final density of 100 Klett units (8–12 h). They were isolated by centrifugation and suspended in 1.5 ml of 100 mM KP_i pH 6.0, 0.5% galactose, 33 μM CCCP. After 10 s, 1 ml of carrier-free $^{35}SO_4^{2-}$ (10 mCi/ml) was added, the suspension was shaken at 30°C for 20 min and then mixed with 30 ml of 10% TCA. The precipitate was washed twice with 30 ml of 10% TCA and once with 30 ml of H_2O. The pellet was agitated with glass beads for 2 min (Ohashi *et al.*, 1982) and extracted at 30°C overnight with 4 ml of 2% SDS, 1% 2-mercaptoethanol, 1 mM EDTA, 0.001% bromothymol blue and sufficient Tris base to adjust the pH to approximate neutrality. The mixture was then diluted with 36 ml of 55.5 mM Tris-Cl pH 7.7, 1.1% Triton X-100, 1 mM EDTA, 0.11 M NaCl. The glass beads were removed by decantation. Solid BSA was dissolved in the supernatant to a final concentration of 10 mg/ml and residual insoluble matter was removed by centrifugation (40 min at 100 000 *g*). The clarified supernatant (total volume 40 ml) usually contained 2 x 10^9 protein-bound c.p.m. The specific radioactivity of labeled yeast proteins was ~10^9 c.p.m./mg.

Initially, the radiolabeled precursor was isolated from the labeled cell extract essentially as described for the unlabeled precursor (cf. above), with three modifications: (i) removal of excess SDS by dialysis was omitted; (ii) the size of the immune column was reduced to 2 ml; and (iii) the order of chromatofocussing and isoelectric focussing was changed in order to remove Nonidet P40 from the final preparation. This procedure yielded precursor that was radiochemically pure and ~30% pure in chemical terms (Figure 1C). Subsequently we adopted a simplified procedure yielding β-subunit precursor that was radiochemically pure but still contained unlabeled mature β-subunit.

No functional difference was detected between the two precursor preparations. In this simplified procedure, the clarified extract of the ^{35}S-labeled *rho*$^-$ cells (40 ml; ~2 x 10^9 protein-bound c.p.m.) was passed through a 5 ml 'pre-column' which was connected in series to a 2 ml immune column. (The pre-column was prepared in the same way as the immune column except that the monoclonal antibody against the β-subunit was replaced by the same weight of non-immune bovine IgG.) The monoclonal antibody column was washed with 10 mg/ml BSA in PBS before use. The labeled yeast proteins were washed into both columns with 10 ml of 1% Triton X-100, 50 mM Tris-Cl, pH 7.7, 1 mM EDTA, 0.3 M NaCl, 0.05% 2-mercaptoethanol at a flow rate of 3.2 ml/h. The pre-column was then disconnected and the immune column was washed successively (flow rate = 3.2 ml/h) with (i) 1% Triton X-100, 50 mM Tris-Cl pH 7.7, 1 mM EDTA, 0.5 M NaCl, 0.05% 2-mercaptoethanol (overnight); (ii) 0.1% cholate, 50 mM Tris-Cl pH 7.7, 1 mM EDTA, 0.3 M NaCl, 0.05% 2-mercaptoethanol (for 8 h); (iii) 2 mg BSA/ml, 20 mM Hepes-KOH pH 7.4, 0.1 M NH_4Cl, 0.05% 2-mercaptoethanol (overnight).

The adsorbed ^{35}S-labeled β-subunit precursor was then eluted with 7.5 M urea, 20 mM Hepes-KOH (pH 7.4), 0.1 M NH_4Cl, 0.05% 2-mercaptoethanol, 2 mg/ml BSA. Fractions of 20 drops each were collected in siliconized glass tubes and counted for ^{35}S; the radioactive fractions (1 x 10^6 c.p.m. total) were pooled; aliquots were frozen and stored at −70°C. Before each experiment, an appropriate aliquot (usually 300 μl) was thawed, transferred to a vertical glass tube (diameter 11 mm; siliconized and then coated with BSA) whose bottom end was sealed with dialysis tubing. The bottom edge of the filled tube was placed into 10% glycerol, 20 mM Hepes-KOH pH 7.4, 0.1 M KCl, 1 mM $MgCl_2$ and dialysis was allowed to proceed for 90 min at +4°C. This was followed by a 90 min dialysis (at +4°C) against 0.6 M sorbitol, 20 mM Hepes-KOH pH 7.4, 0.1 M KCl.

Import of radiochemically pure β-subunit precursor into isolated yeast mitochondria

Import of ^{35}S-labeled β-subunit precursor was measured essentially as described (Gasser *et al.*, 1982); the renatured, radiochemically pure precursor (10 000−14 000 c.p.m.) was incubated for 30 min at 27°C with 250 μg yeast mitochondria, in a final volume of 400 μl containing 0.6 M sorbitol, 1 mM $MgCl_2$, 1 mM ATP, 5 mM GTP, 20 mM Hepes-KOH pH 7.4, 75 mM KCl, 1 mM DTT, 4 units of pyruvate kinase, 5 mM phosphoenolpyruvate and cytosolic fractions as indicated. The mitochondria were then sedimented (Eppendorf microfuge; 10 min at ~12 000 *g*; 4°C) through 1 ml of a cushion of 0.6 M sucrose, 20 mM Hepes-KOH pH 7.4, 2 mM EDTA, 2 mM 1,10-phenanthroline, 1 mM PMSF, 0.1 mM TLCK, resuspended in a minimal volume of the above-mentioned sucrose solution, dissociated in 2% SDS, 1% 2-mercaptoethanol (5 min at 95°C) and analyzed by SDS-10% polyacrylamide gel electrophoresis and fluorography. For assaying polypeptides resistant to externally-added trypsin, mitochondria, precursor and, if present, cytosolic extract were incubated as described above, chilled on ice and incubated for 20 min at 0°C with 125 μg of trypsin/ml. Turkey egg trypsin inhibitor and PMSF were then added to the 2.5 mg/ml and 1 mM, respectively and the mitochondria isolated through a sucrose cushion as described above.

Miscellaneous

Published procedures were used for measuring protein (Lowry *et al.*, 1951), SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels (Douglas and Butow, 1976), immunodecoration of SDS-polyacrylamide gels (Towbin *et al.*, 1979), immunoprecipitation (Gasser *et al.*, 1982), preparation of yeast F_1-ATPase (Douglas *et al.*, 1977), *in vitro* protein synthesis (Pelham and Jackson, 1976), isolation of yeast RNA (Maccecchini *et al.*, 1979), raising of rabbit antisera against cytochrome b_2 (Daum *et al.*, 1982a), isolation of partially purified matrix protease (Böhni *et al.*, 1983), fluorography of dried SDS-polyacrylamide gels (Chamberlain, 1979) and silver staining of proteins on polyacrylamide gels (Wray *et al.*, 1981).

[^{35}S]Methionine (>1000 Ci/mmol) and carrier-free $^{35}SO_4^{2-}$ were from Amersham (UK); protein A was from Pharmacia (Sweden).

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