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Disruption of the outer membrane restores protein import to trypsin-treated yeast mitochondria

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Treatment of isolated yeast mitochondria with high levels (1 mg/ml) of trypsin severely inhibits protein import but does not destroy the integrity of the outer membrane or abolish mitochondrial energy coupling. If the outer membrane of these trypsin-inactivated mitochondria is disrupted by osmotic shock, the resulting mitoplasts are again able to import proteins. Protein import into mitoplasts, like that into intact mitochondria, is energy-dependent; however, whereas import into mitochondria is inhibited by antibody against 45-kd proteins of the outer membrane [Ohba and Schatz, *EMBO J.*, 6, 2109–2115 (1987)], import into mitoplasts not affected by this antibody. Protein import into mitoplasts appears to bypass one or more steps normally occurring at the mitochondrial surface.

Key words: protein import/yeast mitochondria/mitoplasts/trypsin

Introduction

Proteins transported from the cytoplasm into the mitochondrial matrix must pass two mitochondrial membranes. How is this accomplished? There is mounting evidence that transport is initiated by one or more outer membrane protein(s) which may function as 'import receptor(s)' (Hay *et al.*, 1984; Zwizinski *et al.*, 1983, 1984; Riezman *et al.*, 1983b; Ohba and Schatz, 1987). Passage across both mitochondrial membranes may then occur at 'contact sites' in which the two membranes are in close contact, or even fused with each other (Kellems *et al.*, 1975; Schleyer and Neupert, 1985). This proposed mechanism does not exclude, however, that each of the two mitochondrial membranes contains a separate translocation machinery. In intact mitochondria, these two devices might operate as a closely coupled unit which would make it difficult to analyze each of them separately.

In order to learn whether the mitochondrial inner membrane contains a distinct protein import system, we performed a two-step experiment. First, we inactivated the translocation sytem of the outer membrane by treating the surface of intact mitochondria with high levels of trypsin. Second, we disrupted the outer membrane of these trypsin-treated mitochondria by osmotic shock and checked whether the resulting 'mitoplasts' could again import proteins. This was indeed the case. Mitoplasts may thus offer unique possibilities for dissecting the steps by which proteins are transported across the two mitochondrial membranes.

Results

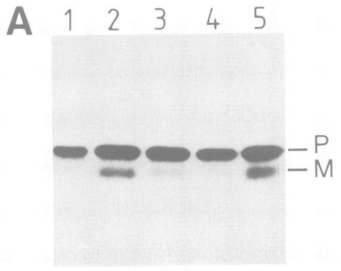
Trypsin-treatment of intact yeast mitochondria blocks protein import, but does not disrupt the outer membrane

Several laboratories, including ours, had already reported that treatment of mitochondria with different proteases inactivated import of *in vitro*-synthesized mitochondrial precursor proteins into these organelles (cf. references above). In these earlier experiments, import was studied with radiolabelled authentic precursor proteins that had been synthesized from total cellular mRNA in a nuclease-pretreated reticulocyte lysate. Figure 1A shows that trypsin-treatment of intact mitochondria also inhibits import of a purified artificial precursor protein; this artificial precursor is a fusion protein which contains the first 22 residues of the cytochrome oxidase subunit IV precursor (a protein imported into the matrix) fused to mouse dihydrofolate reductase (DHFR, a cytosolic enzyme). *In vitro* and *in vivo*, this fusion protein (termed pCOXIV–DHFR) behaves like an authentic mitochondrial precursor protein (Hurt *et al.*, 1984, 1985). Trypsin-treatment also inhibits mitochondrial import of the same protein synthesized by coupled transcription/translation *in vitro* (Figure 1B) or of *in vitro*-synthesized authentic cytochrome oxidase subunit IV precursor (Figure 1C). The fluorograms of panels A–C are quantified in panel F.

Effective inhibition of mitochondrial protein import required much higher concentrations of trypsin than in our earlier work (Riezman *et al.*, 1983b). The reason for this is not clear. In our hands, analysis of commercially available trypsin samples by SDS–polyacrylamide gel electrophoresis always revealed contaminating protein bands (not shown) which might conceivably influence the effect of trypsin on the mitochondrial surface. Also, import of the precursors used here is less sensitive to trypsin than that of the F_1 beta-subunit precursor (not shown) which had been extensively studied in our earlier work. While we cannot pinpoint the reason for the quantitative discrepancy to our earlier experiments, the conclusion remains the same: protein import into isolated yeast mitochondria can be inhibited by treating the intact organelles with trypsin. Different precursors were inhibited to different degrees, even if they shared the same presequence. The inhibition shown in Figure 1 reflected the action of trypsin (rather than that of some contaminating proteases or lipases) since it was abolished by prior addition of soybean trypsin inhibitor. These 'mock-treated' samples are shown in lanes 5.

In order to exclude that the inhibition of precursor import into mitochondria merely reflected degradation of the added precursor by residual traces of trypsin, the amount of non-imported precursor in the supernatants after removal of mitochondria was analyzed. No degradation of the pCOXIV–DHFR fusion protein was detected (Figure 1H and I). While there was some degradation of the subunit IV precursor (Figure 1K), import of this precursor was the least sensitive to trypsin-pretreatment of the mitochondria (compare Figure 1A and B with Figure 1C). Thus, inhibition of import is not an artefact resulting from degradation of added precursor.

Exposure of mitochondria to the rather high trypsin concentrations used here did not disrupt the mitochondrial membranes: cytochrome b_2 (b_2; a soluble enzyme of the intermembrane space) and citrate synthase (CS; a soluble enzyme of the matrix) remained inaccessible to the externally added trypsin (Figure 1,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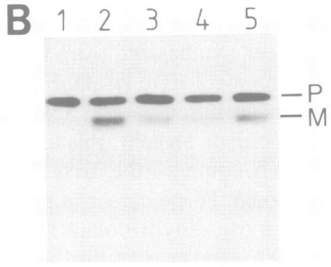
1 2 3 4 5
P
M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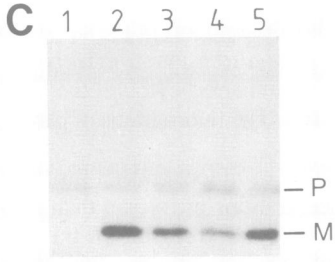
1 2 3 4 5
P
M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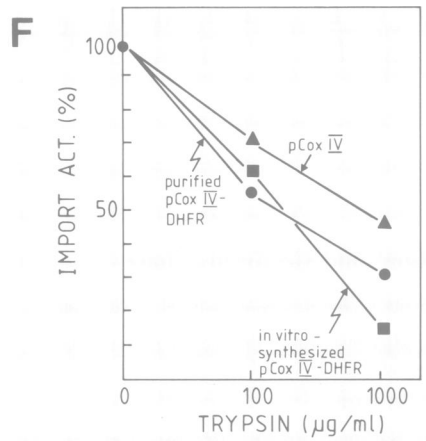
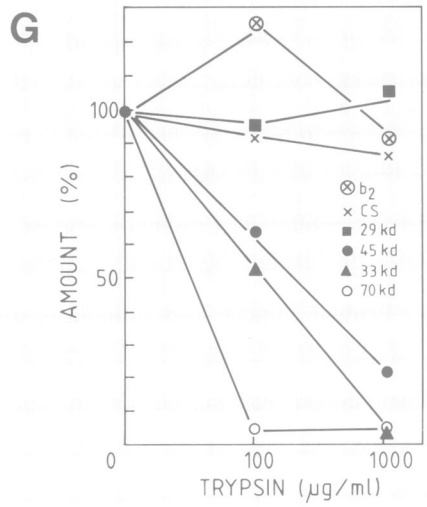
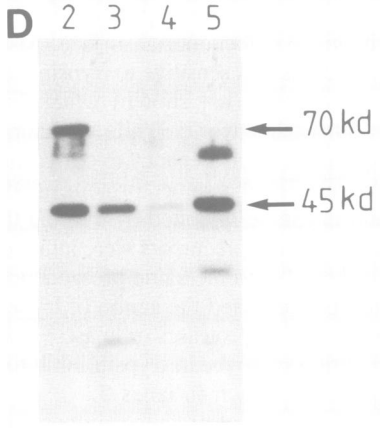
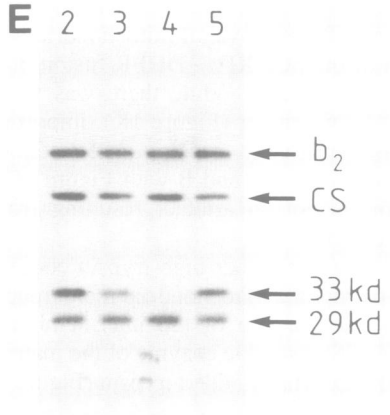
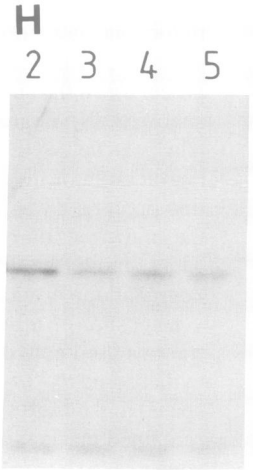
1 2 3 4 5
P
M

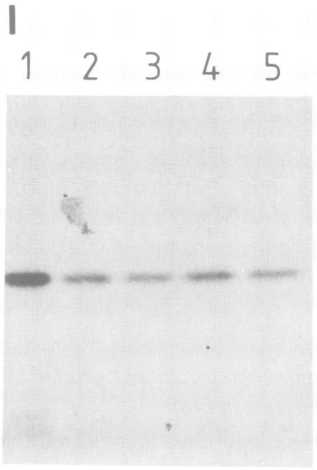

F
100
50
0
100
1000
IMPORT ACT. (%)
TRYPSIN (μg/ml)
pCox IV
purified pCox IV-DHFR
in vitro-synthesized pCox IV-DHFR

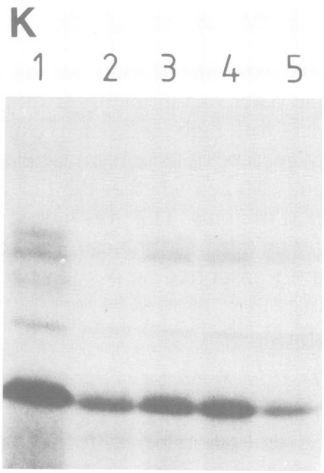

G
100
50
0
100
1000
AMOUNT (%)
TRYPSIN (μg/ml)
b2
CS
29 kd
45 kd
33 kd
70 kd


D
2 3 4 5
70 kd
45 kd


E
2 3 4 5
b2
CS
33 kd
29 kd


H
2 3 4 5


I
1 2 3 4 5


K
1 2 3 4 5

panels D–G). Also, the trypsin-treated mitochondria still maintained a potential across the inner membrane and exhibited respiratory control (Table I).

The different outer membrane proteins responded to the proteolytic treatment as expected from our earlier work (Riezman *et al.*, 1983a). The 70-kd outer-membrane protein was very sensitive to protease; even 'mock-treatment' in the presence of trypsin inhibitor affected it, generating the cytosolically exposed 60-kd fragment (Figure 1D, lane 5) of this protein (Hase *et al.*, 1984). The 45-kd and 33-kd proteins were partly digested by 0.1 mg/ml trypsin and almost completely digested by 1 mg/ml trypsin whereas the pore-forming 29-kd protein was resistant to even 1 mg/ml trypsin.

Osmotic disruption of the outer membrane restores protein import to trypsin-treated mitochondria

Mitoplasts (mitochondria whose outer membrane had been disrupted) still import proteins (Daum *et al.*, 1982b; van Loon and Schatz, 1987). This is confirmed in Figure 2. Import of the pCOXIV–DHFR fusion protein into mitoplasts resembled import into mitochondria in several respects: it was equally fast (Figure 2B, left part), energy-dependent, accompanied by proteolytic removal of the attached presequence, and inhibited by the DHFR-ligand, methotrexate (Figure 2A, upper part; see also Eilers and Schatz, 1986).

The immune blots shown in Figure 2C confirmed that the osmotic-shock procedure had converted at least two-thirds of the mitochondria to mitoplasts: upon sedimentation of the mitoplasts, almost 70% of the soluble intermembrane space enzyme, cytochrome b_2 was recovered in the supernatant (lane 3S and open bar below it) whereas >95% of cytochrome b_2 co-sedimented with the mitochondria (lane 2P and cross-hatched bar below it).

While the combined data of Figure 2 made it unlikely that the observed protein import into our mitoplast preparation was merely caused by residual mitochondria, it did not rigorously exclude this possibility. Even if it could have been excluded, the pathway of proteins into these mitoplasts might still be the same as that into intact mitochondria: since mitoplasts prepared by physical methods still contain most of their outer membrane adhering to contact sites on the inner membrane (Hackenbrock, 1968; Greenawalt, 1979), and since these contact sites might be the entry point of proteins into mitochondria (cf. above), osmotic disruption of the outer membrane might not have altered the mitochondrial machinery for importing proteins.

To eliminate this uncertainty, we prepared mitoplasts from mitochondria whose protein import system had been inactivated by trypsin treatment. These mitochondria still retained all of their cytochrome b_2 (Figure 2C, lane 4P and cross-hatched bar below it), but imported the pCOXIV–DHFR fusion protein eight times more slowly than the untreated mitochondria (cf. lanes 4 in the panels labelled 'MITOCHONDRIA' of Figure 2A and the 5-min time points in the traces marked by solid circles in Figure 2B). However, mitoplasts prepared from these inactivated mitochondria imported the fusion protein nearly as rapidly as untreated mitochondria, or as mitoplasts derived from untreated mitochondria (cf. lanes 4 in the panels labelled 'MITOPLASTS' in Figure 2A and the traces marked by open squares in Figure 2B). The immune blots of Figure 2C (lanes 5 and the bars below) confirmed that almost 90% of the trypsin-inactivated mitochondria had been converted to mitoplasts. This result suggests that import into mitoplasts is qualitatively different from that into intact mitochondria.

Table I. Mitochondria treated with high concentrations of trypsin retain energy coupling

Treatment of mitochondria	Membrane potential (arbitrary units)		Respiratory control ratio
	Expt 1	Expt 2	
None	31 (100%)	10 (100%)	1.8 (100%)
0.1 mg trypsin/ml	28 (90%)	9 (90%)	1.7 (94%)
1 mg trypsin/ml	32 (103%)	9 (90%)	1.5 (83%)
1 mg trypsin/ml and 10 mg trypsin inhibitor/ml	n.d.	11 (110%)	n.d.

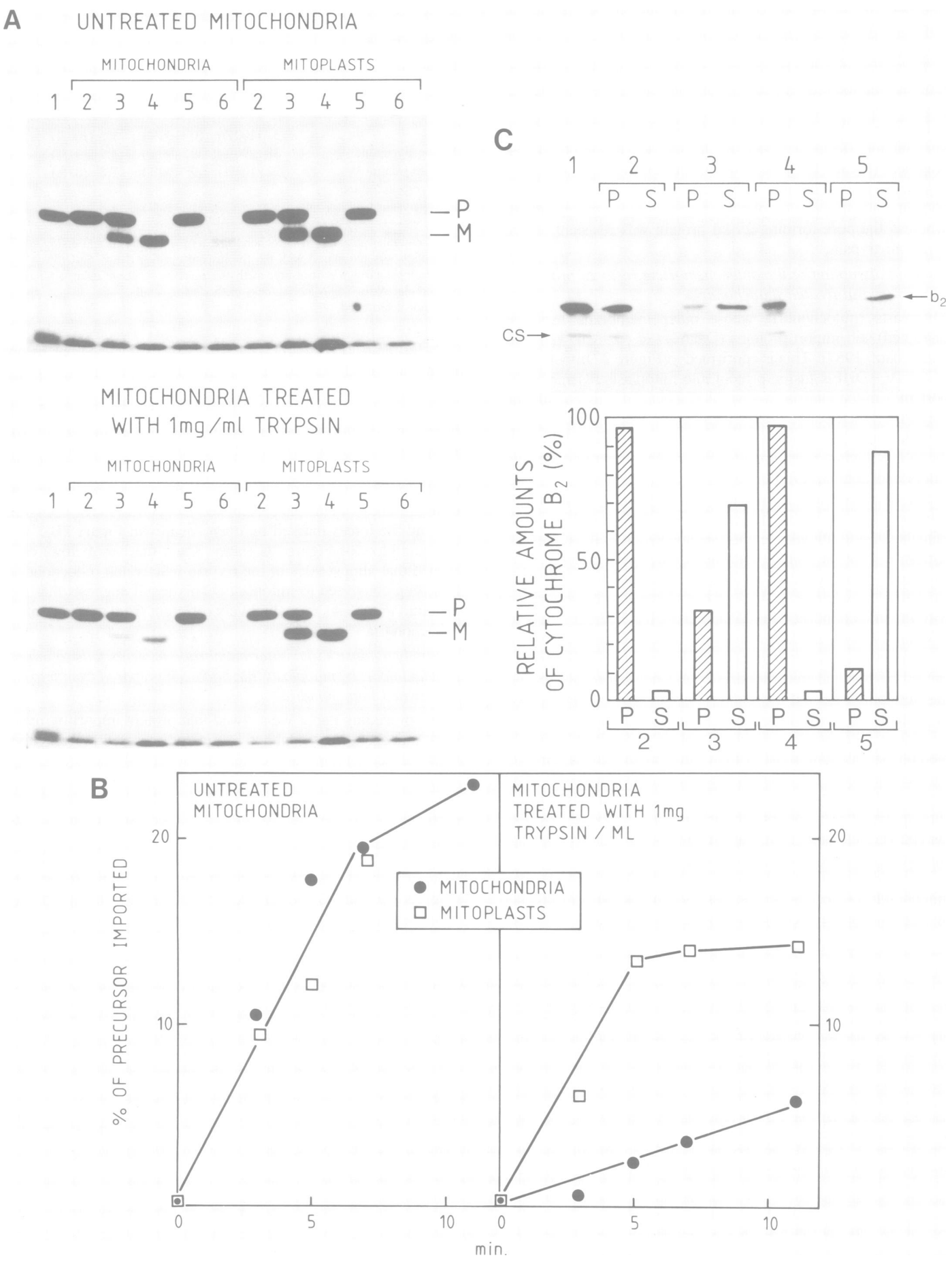
The membrane potential of mitochondria energized by ATP and endogenous respiration was monitored with the fluorescent cyanine dye di-S_3-C(5); respiratory control was measured with succinate as substrate using a Clark oxygen electrode. See Materials and methods for details.
n.d., not determined.

Antibody against 45-kd outer membrane proteins fails to inhibit protein import by mitoplasts that had been prepared from trypsin-inactivated mitochondria

In the preceding paper we showed that protein import by intact yeast mtiochondria is inhibited by antibodies raised against either the total outer membrane or against several 45-kd proteins of the outer membrane (Ohba and Schatz, 1987). We interpreted this result as evidence for a mitochondrial surface protein that participates in the translocation of precursor proteins into mitochondria.

Since 45-kd proteins cross-reacting with this antibody are largely degraded by treating mitochondria with 1 mg trypsin/ml (Figure 1D and G), we checked whether protein import into mitoplasts derived from trypsin-treated mitochondria had become

Fig. 1. Treatment of mitochondria with high concentrations of trypsin inhibits import of proteins without disrupting the outer membrane. Isolated yeast mitochondria (100 μg protein) were treated with trypsin (either 0.1 or 1 mg/ml); trypsin was then inhibited and the mitochondria were tested for their ability to import the ^{35}S-labelled proteins mentioned below under conditions in which the amount of import was proportional to time and the amount of mitochondria (see Materials and methods). Control experiments also ascertained that the proteolytic processing of the precursor proteins by isolated mitochondria reflected import: processing was completely blocked by uncoupling the mitochondria with valinomycin and K^+ (cf. Figure 2). Finally, mitochondria were re-isolated and analyzed for imported radiolabelled proteins by SDS–polyacrylamide gel electrophoresis and fluorography. Aliquots of the supernatants were also analyzed (see **H–K**). **A** Purified fusion protein (1.5×10^5 c.p.m.) containing the first 22 residues of the cytochrome oxidase subunit IV precursor attached to mouse DHFR (pCOXIV–DHFR). **B** pCOXIV–DHFR synthesized by coupled transcription/translation in a nuclease-pretreated reticulocyte lysate. **C** Authentic precursor of cytochrome oxidase subunit IV synthesized in a reticulocyte lysate. **Lane 1**, 10% of the amount of radiolabelled protein added to each mitochondrial sample; **lane 2**, incubation with untreated mitochondria; **lane 3**, incubation with mitochondria that had been treated with 0.1 mg trypsin/ml; **lane 4**, mitochondria treated with 1 mg trypsin/ml; **lane 5**, mitochondria treated with 1 mg/ml trypsin in the presence of 10 mg soybean trypsin inhibitor (STI)/ml ('mock-treated'). P and M, uncleaved and cleaved (= imported) precursor or fusion protein. **D** and **E** Separate aliquots of the mitochondrial samples that had been treated and then tested for protein import as described above (**panels A–C**) were analyzed for intactness of their outer membrane by immune blotting with the following antisera. **D**, A mixture of antisera against the 70-kd and 45-kd outer membrane proteins; **E**, a mixture of antisera against the 33-kd outer membrane protein, the 29-kd outer membrane protein, cytochrome b_2 (b_2; soluble intermediate space marker) and citrate synthase (CS; soluble matrix marker). Lanes correspond to those of **panels A–C**. **F** and **G** The results of the import experiments shown in **panels A–C** and of the immune blotting experiments shown in **panels D** and **E** were quantified by densitometry. **H, I** and **K** 25% of the supernatants, from the samples shown in **panels A, B** and **C** respectively, were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. Numbering of the lanes are the same as those in **panels A–C**.


A
UNTREATED MITOCHONDRIA
MITOCHONDRIA
MITOPLASTS
1 2 3 4 5 6 2 3 4 5 6
P
M
MITOCHONDRIA TREATED WITH 1mg/ml TRYPSIN
MITOCHONDRIA
MITOPLASTS
1 2 3 4 5 6 2 3 4 5 6
P
M
C
1 2 3 4 5
P S P S P S P S
b2
CS
RELATIVE AMOUNTS OF CYTOCHROME B2 (%)
100
50
0
P S P S P S P S
2 3 4 5
B
UNTREATED MITOCHONDRIA
MITOCHONDRIA TREATED WITH 1mg TRYPSIN / ML
MITOCHONDRIA
MITOPLASTS
% OF PRECURSOR IMPORTED
20
10
0 5 10 0 5 10
min.

independent of 45-kd outer membrane proteins. This was indeed the case. Protein import into mitoplasts from trypsin-treated mitochondria (Figure 3, lane 1) was completely insensitive to antibody against outer membrane 45-kd proteins (lane 4) whereas import into untreated mitochondria was sensitive to these antibodies. Figure 3 also confirms that the trypsin-treated mitochondria could no longer import proteins. In these experiments, only a small fraction of the added precursor was imported since import was only allowed to occur for 5 min; this short duration was chosen to ensure that import was linear with time even with the more fragile mitoplasts (cf. Figure 2B). Lack of inhibition by the anti-45-kd antibodies further supports the view that protein import into mitoplasts derived from trypsin-inacivated mitochondria is qualitatively different from that into untreated mitochondria.

Discussion

Here we show that protein import into isolated yeast mitochondria is almost totally inactivated by trypsin treatment and that it can be subsequently restored by osmotic disruption of the outer membrane. This result was quite unexpected and does not readily fit into current views on how proteins are transported across the two mitochondrial membranes (Hay *et al.*, 1984). It is now widely assumed that import occurs at contact sites between the two mitochondrial membranes. This view stemmed from an early observation that, under certain experimental conditions, cytoplasmic ribosomes were bound to the yeast mitochondrial surface at sites where the two mitochondrial membranes appeared to be in close apposition (Kellems *et al.*, 1975). More recent evidence for the functional significance of such 'contact sites' came from the demonstration that precursor proteins partially imported into mitochondria can span both membranes (Schleyer and Neupert, 1985). However, neither of these observations prove that contact sites between the two mitochondrial membranes are obligatory entry points for cytoplasmically synthesized mitochondrial proteins.

At first sight our data appear to favour a model according to which proteins destined for the matrix space are sequentially transported across the outer and then across the inner membrane. The outer membrane with its putative 'import receptor(s)' (cf. above) might select mitochondrial precursor proteins from the cytosol and transport these into the intermembrane space. In a second step, the inner membrane might then complete the translocation process via its own transport machinery. According to this model, import into the mitoplasts from trypsin-inactivated mitochondria would be a partial reaction which bypasses the first step at the outer membrane.

However, the present data do not rule out that protein import into the mitochondrial matrix occurs through 'contact sites'. These sites might only be generated upon binding of a mitochondrial precursor protein to a receptor-like component on the surface of the outer membrane. Alternatively, import into mitoplasts might occur by a pathway that is not used with intact mitochondria. However, our data suggest that the pathway into mitoplasts shares at least some steps with the import pathway operative *in vivo*: translocation into mitoplasts is accompanied by proteolytic cleavage of the presequence and dependent on an energized inner membrane. Since import of the DHFR-containing fusion protein is inhibited by methotrexate, it requires at least partial unfolding of the protein, similar to the situation previously observed with mitochondria (Eilers and Schatz, 1986). Further analysis of protein import into these mitoplasts should be of considerable interest.

Materials and methods

Trypsin treatment of mitochondria

Mitochondria were isolated from the *Saccharomyces cerevisiae* strain D273-10B (ATCC 25657) grown on semi-synthetic medium containing 2% lactate (Daum *et al.*, 1982a). They were suspended in 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4 at 10 mg/ml. TPCK-treated trypsin (see Ohba and Schatz, 1987) was added to 0.1 or 1.0 mg/ml and the mixture was incubated for 30 min at 0°C. Proteolysis was stopped by adding a 10-fold excess (w/w) of soybean trypsin inhibitor (STI) and incubation for an additional 10 min at 0°C. The mitochondria were re-isolated by centrifugation, suspended in 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4, 1 mg/ml STI and re-sedimented through 0.6 ml of 0.6 M sucrose, 20 mM Hepes–KOH pH 7.4, 1 mg/ml STI and suspended in 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4, 1 mg/ml STI to ~10 mg/ml. 'Untreated' mitochondria were carried through exactly the same procedure except that addition of trypsin was omitted.

Preparation of mitoplasts

Untreated mitochondria or mitochondria treated with 1 mg/ml trypsin (cf. preceding section) were diluted with 9 vol 20 mM Hepes–KOH pH 7.4, 1 mg/ml STI and incubated for 20 min at 0°C to disrupt the outer membrane. The resulting mitoplasts were re-isolated by centrifugation for 10 min at 12 000 *g* and suspended in 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4, 1 mg/ml STI. To check the effectivenss of conversion to mitoplasts, the supernatant from this centrifugation was analyzed for cytochrome b_2 by immune blotting to assess release of this soluble intermembrane space enzyme (see Figure 2C). As a control, mitochondria were treated identically except that the initial mitochondrial suspension was diluted with 9 vol of 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4, 1 mg/ml STI. These control mitochondria were used as 'MITOCHONDRIA' in the experiment depicted in Figures 1–3.

Antibody treatment

Antibody treatment of mitochondria or mitoplasts was done as described in the accompanying paper (Ohba and Schatz, 1987) except that 100 μg/ml trypsin inhibitor was included during incubations.

Protein import into isolated mitochondria and mitoplasts

The assay mixture (total volume 0.2 ml) contained 100 μg of either mitochondria or mitoplasts, an ATP-regenerating system (5 mM phosphoenolpyruvate, 1 mM ATP, 0.6 unit pyruvate kinase, 1 mg $MgCl_2$), 0.6 M mannitol, 20 mM Hepes–KOH pH 7.4, 5 mM GTP, 2.5 mg/ml bovine serum albumin, 1 mg/ml

Fig. 2. Disruption of the outer membrane restores protein import to mitochondria that had been pretreated with high (1 mg/ml) trypsin concentrations. Yeast mitochondria (either untreated or treated with 1 mg/ml trypsin) and mitoplasts derived from these two types of mitochondria were prepared as described in Materials and methods. **A** Aliquots (100 μg) of the pelleted mitochondria or mitoplasts were assayed for their ability to import the *in vitro*-synthesized pCOXIV–DHFR fusion protein. Import was for 10 min at 30°C. **Lane 1**, 10% of the radiolabelled fusion protein added to each of the import assays; **lane 2**, after incubation with mitochondria or mitoplasts that had been de-energized with 100 μM valinomycin and K^+, **lane 3**, after incubation with energized mitochondria or mitoplasts (import); **lane 4**, same as **lane 3**, but organelles treated with 250 μg proteinase K/ml after import; **lane 5**, same as **lane 3**, but import in the presence of 50 nM methotrexate; **lane 6**, same as **lane 5**, but organelles treated with 250 μg proteinase K/ml after import. Samples were analyzed by SDS–polyacrylamide gel electrophoresis and fluorography. P and M, uncleaved and cleaved (= imported) fusion protein respectively. **B** Mitochondria (untreated or treated with 1 mg trypsin/ml) and mitoplasts derived from them were allowed to import *in vitro*-synthesized pCOXIV–DHFR fusion protein for the times shown on the abscissa. Import was then assayed as described above and quantified by densitometry of the labelled band corresponding to the cleaved precursor. **C** Conversion of the mitochondria to mitoplasts was assessed by immune blotting to measure release of the intermembrane space marker, cytochrome b_2 (b_2) and retention of the soluble matrix marker, citrate synthase (CS). Each gel lane was loaded with an amount of fraction derived from 100 μg of mitochondria. **Lane 1**, original mitochondria; **lane 2**, mitochondrial pellet (P) and supernatant (S) from mitochondria incubated without trypsin; **lane 3**, mitoplast pellet and supernatant from mitochondria incubated without trypsin; **lane 4**, mitochondrial pellet and supernatant from trypsin-treated mitochondria; **lane 5**, mitoplast pellet and supernatant from trypsin-treated mitochondria. The immune signal of cytochrome b_2 was quantified and the data are given as bars in the lower half of **panel C**.

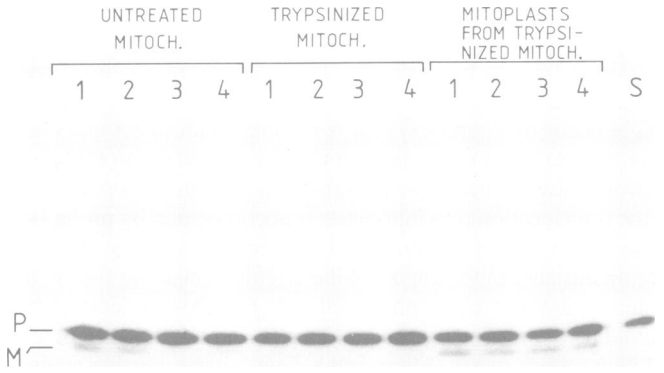


Fig. 3. The restored protein import by mitoplasts derived from trypsin-inactivated mitochondria is resistant to IgG raised against total outer membrane or against outer membrane 45-kd proteins. Aliquots (100 μg protein) of untreated yeast mitochondria, yeast mitochondria pretreated with 1 mg trypsin/ml, and of mitoplasts derived from the trypsin-treated mitochondria were incubated for 30 min at 0°C in a final volume of 50 μl without IgG, with 750 μg non-immune IgG, with 750 μg IgG against total outer membrane, or with 750 μg IgG raised against the 45-kd proteins of the outer membrane. They were then directly analysed for import of the *in vitro*-synthesized pCOXIV−DHFR fusion protein without prior re-isolation. Import was allowed to proceed for only 5 min at 30°C; under these conditions import is linear with time, with mitochondria and with mitoplasts. Finally, the particles were re-isolated and analysed by SDS−polyacrylamide gel electrophoresis and fluorography. **Lane 1**, no IgG; **lane 2**, non-immune IgG; **lane 3**, IgG against total outer membrane; **lane 4**, IgG against 45-kd proteins of the outer membrane. S, 10% of the radiolabelled fusion protein that was added to each import assay; P and M, uncleaved and cleaved (= imported) fusion protein.

STI, 0.1 mM phenylmethylsulfonyl fluoride and 7.5−10 μl of precursor solution. The precursor was either highly purified, ^{35}S-labelled pCOXIV−DHFR fusion protein (1.5 × 10^5 c.p.m. ^{35}S in 10 μl; Eilers and Schatz, 1986) or a [^{35}S]methionine-labelled *in vitro* transcription/translation mixture which had been programmed by the plasmid-borne genes for either pCOXIV−DHFR or for the authentic cytochrome oxidase subunit IV precursor (plasmid pDS 5/2 -1-COXIV; Hurt *et al.*, 1984). Import was allowed to occur at 30°C for only 5−10 min to ensure linearity with time. It was stopped by adding 0.2 ml of ice-cold 0.6 M mannitol, 20 mM Hepes−KOH pH 7.4, 20 mM valinomycin. The organelles were re-isolated by centrifugation and analyzed for imported radiolabelled proteins by SDS−polyacrylamide gel electrophoresis and fluorography (Hurt *et al.*, 1984). Control experiments (not shown) confirmed that the amount of imported proteins was a linear function of the amount of mitochondria or mitoplasts between 30 and 150 μg.

Measurement of energy coupling and membrane potential

To assess mitochondrial integrity, respiratory control was measured with a Clark oxygen electrode. The reaction mixture (1 ml) consisted of 200 μg mitochondria, 0.6 M mannitol, 20 mM Hepes−KOH pH 7.4, 20 mM KPi pH 7.4, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mg/ml fatty acid-free bovine serum albumin and 10 mM succinate pH 7.4. The rate of oxygen consumption was measured before and after the addition of 5 μl of 20 mM ADP. The respiratory control index is defined as the rate of respiration upon addition of ADP divided by the rate of respiration before addition of ADP.

To monitor the membrane potential, the potential-dependent fluorescence of di-S_3-C(5) (Sims *et al.*, 1974) in the presence of mitochondria was measured with a Schoeffel RRS 1000 recording fluorimeter (excitation at 620 nm and emission at 670 nm). The reaction mixture (2.5 ml) contained 2 μM of di-S_3-C(5), 40 mM KCl, 1 mM ATP, 1 mM $MgCl_2$, 5 mM phosphoenolpyruvate and 0.6 unit of pyruvate kinase. The mitochondrial suspension (250 μg mitochondria in 25 μl) and 5 μl of 1 mM valinomycin in ethanol were added successively. Fluorescence emission was measured at room temperature. The change in fluorescence intensity between these two additions was taken as a measure of the membrane potential.

Miscellaneous

Immune blotting was carried out as described (Haid and Suissa, 1983) with antibodies against outer membrane proteins (Riezman *et al.*, 1983a). Where indicated, fluorographs were quantified by densitometric scanning. Protein was measured by the BCA-procedure described by Pierce Chemical Co., Rockford, IL (USA).

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