Disrupted Yeast Mitochondria Can Import Precursor Proteins Directly Through Their Inner Membrane

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Abstract. Import of precursor proteins into the yeast mitochondrial matrix can occur directly across the inner membrane. First, disruption of the outer membrane restores protein import to mitochondria whose normal import sites have been blocked by an antibody against the outer membrane or by a chimeric, incompletely translocated precursor protein. Second, a potential- and ATP-dependent import of authentic or artificial precursor proteins is observed with purified inner membrane vesicles virtually free of outer membrane components. Third, import into purified inner membrane vesicles is insensitive to antibody against the outer membrane. Thus, while outer membrane components are clearly required in vivo, the inner membrane contains a complete protein translocation system that can operate by itself if the outer membrane barrier is removed.

MITOCHONDRIA and chloroplasts import most of their proteins as precursors which are synthesized in the cytoplasm. Many of these precursors must be translocated across two membranes to reach their final location in the matrix or the stroma, respectively (Attardi and Schatz, 1988). There is mounting evidence that passage across both organelle membranes occurs through sites where the two membranes are closely apposed (Hackenbrock, 1968; Kellems et al., 1975; Schwaiger et al., 1987; Pain et al., 1988; Vestweber and Schatz, 1988). The mechanism by which this import occurs is not known. The "contact sites" might represent an integrated translocation device in which outer membrane components are mechanistically involved in transporting a protein across the outer as well as inner membrane. It is also possible, however, that outer membrane components are only involved in moving precursors across the outer membrane, whereas transport across the inner membrane is mediated exclusively by components of the inner membrane. The former model was favored by Schwaiger et al. (1987); they blocked the import activity of isolated *Neurospora* mitochondria by a mild protease treatment and found that subsequent disruption of the outer membrane did not restore import. The opposite result was reported for isolated yeast mitochondria: import was blocked by trypsin, but could be reactivated by disrupting the outer membrane (Ohba and Schatz, 1987*a*). The results with yeast suggested, but did not prove, that the inner membrane contains all the components necessary for translocating precursor proteins and that removal of the outer membrane barrier bypasses the need for outer membrane components in translocating precursor proteins into the matrix space.

The results reported in this paper strongly reaffirm the second model: they show that the inner membrane, by itself, can translocate precursors if it is given direct access to a suitable precursor protein.

Materials and Methods

Import of Precursor Proteins into Mitochondria

Mitochondria were prepared from the *Saccharomyces cerevisiae* strain D273-10B; (25657; American Type Culture Collection, Rockville, MD) as described (Daum et al., 1982). Import experiments were carried out at 28–30°C (unless otherwise noted) for the indicated times either with the purified cytochrome oxidase subunit IV presequence-dihydrofolate reductase (pCOXIV-DHFR)[1] fusion protein (Eilers and Schatz, 1986) or with pCOXIV synthesized by transcription/translation (Hurt et al., 1984). The import buffer contained 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 40 mM KCl, 8 mM unlabeled methionine, 1 mM DTT, and 10 mM $MgCl_2$. Energy was supplied by an ATP-regenerating system (Hurt et al., 1984) or only by the oxidation of succinate (Eilers et al., 1987), as indicated. ATP depletion of reticulocyte lysates was performed with apyrase (grade VI; Sigma Chemical Co., St. Louis, MO; Pfanner and Neupert, 1986). Inner membrane vesicles were energized by 10 mM succinate, 10 mM L-malate, 3 mM ascorbate, and 0.5 mg horse heart cytochrome *c*/ml. Whenever import activities of mitochondria and mitoplasts were compared, both types of particles were adjusted to the same final protein concentration (0.25–0.5 mg/ml), except where noted otherwise.

Protease Treatment of Mitochondria and Preparation of Mitoplasts

Treatment of intact mitochondria with 1 mg trypsin/ml and subsequent generation of mitoplasts (mitochondria whose outer membrane is disrupted) were carried out as described by Ohba and Schatz (1987*a*). Trypsin-treated mitochondria, mitoplasts, and untreated mitochondria were suspended to a protein concentration of 5–10 mg protein/ml in 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, 1 mg soybean trypsin inhibitor/ml, and 0.1 mg BSA/ml.

For treating mitochondria or mitoplasts with proteinase K, the particles were suspended to 5 mg protein/ml in the buffer described in the preceding paragraph and incubated for 20 min on ice with the indicated concentrations of proteinase K. Digestion was terminated by diluting the suspension with an equal volume of import buffer containing 1 mM PMSF.

1. *Abbreviations used in this paper*: BPTI, bovine pancreatic trypsin inhibitor; COXIV, cytochrome oxidase subunit IV; DHFR, dihydrofolate reductase; pCOXIV, precursor to cytochrome oxidase subunit IV.

Preparation of Inner Membrane Vesicles

Yeast mitochondria were prepared from a 10-liter culture as mentioned above except that the homogenization buffer (Daum et al., 1982) was supplemented with 10 mM EDTA. The yield of mitochondria was usually 150–200 mg protein. 100 mg of mitochondria was suspended to 10 mg/ml in EDTA-free homogenization buffer and allowed to swell by diluting with 9 vol of 20 mM Hepes-KOH, pH 7.4, 1 mM PMSF, 0.5 mM EDTA and incubating for 30 min on ice. The suspension was then mixed with 0.33 vol of 1.8 M sucrose (to yield a final sucrose concentration of 0.45 M) and incubated for 10 min on ice. The suspended particles (~133 ml) were disrupted by sonication (macrotip; Heat Systems-Ultrasonics, Inc., Farmingdale, NY; 90 s at 80% duty cycle) in an ice bath. Residual intact mitochondria and large fragments were sedimented (at 32,000 *g* for 20 min at 4°C), and submitochondrial particles were collected by centrifugation at 200,000 *g* for 45 min at 4°C. They were resuspended in 5 mM Hepes-KOH, pH 7.4, 10 mM KCl in a final volume of 600 μl to 7–10 mg/ml, and two 300-μl aliquots were layered onto linear sucrose gradients (14 ml/gradient; 0.85–1.6 M sucrose in 10 mM KCl, 5 mM Hepes-KOH, pH 7.4). The gradient was centrifuged at 100,000 *g* for 16 h at 4°C. The densest of the three bands (Pon, L., manuscript in preparation) was collected with a syringe and dialyzed overnight at 4°C against 0.6 M sorbitol, 20 mM KPi, pH 7.4.

Measurement of Membrane Potential

The potential measurements were carried out with the potential-sensitive dye (3,3')-dipropylthiocarbocyanine iodide, (Sims et al., 1974). Excitation was at 620 nm and emission at 670 nm. All incubations were at room temperature in 10 mM $MgCl_2$, 0.5 mM EDTA, 20 mM KPi, pH 7.4, 0.6 M sorbitol, and 1 mg/ml BSA. A 2-mM stock solution of the dye in ethanol was diluted 1,000-fold into the incubation mixture. The final concentration of inner membrane protein was ~8–12 μg/ml.

Miscellaneous

The potential-sensitive dye was obtained from Molecular Probes, Inc. (Junction City, OR). The anti-outer membrane antibodies used in this study had been characterized earlier (Daum et al., 1982; Riezman et al., 1983*a*,*b*; Ohba and Schatz, 1987*b*). Synthesis of the pDV12–bovine pancreatic trypsin inhibitor (BPTI) chimeric precursor was carried out as described by Vestweber and Schatz (1988). Protein was assayed by the "BCA"-procedure (technical bulletin; Pierce Chemical Co., Rockford, IL).

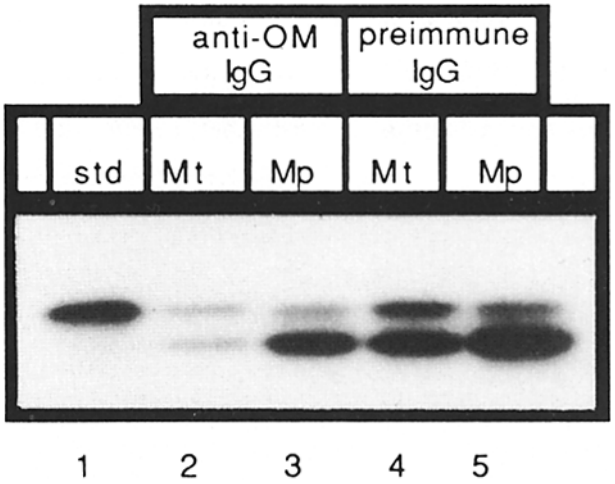

Figure 1. Disruption of the outer membrane restores protein import to mitochondria pretreated with antibody to outer membrane. Yeast mitochondria were pretreated for 30 min at 0°C either with preimmune IgG (2.3 mg/mg mitochondrial protein) or with anti–outer membrane IgG (2.1 mg/mg). Each sample was then divided into two aliquots. One aliquot was diluted ninefold with 0.6 M sorbitol, 20 mM Hepes-KOH, pH 7.4, to maintain mitochondrial integrity; the other aliquot was diluted ninefold with 20 mM Hepes-KOH, pH 7.4, to disrupt the outer membrane. After a 20-min incubation at 0°C, all samples were reisolated by centrifugation at 15,000 *g* for 10 min and tested for their ability to import the purified radiolabeled pCOXIV-DHFR precursor protein. Import was for 8 min at 30°C with 140 μg of particle protein per assay. Import was stopped by adding valinomycin to 10 μg/ml. Samples were analyzed by SDS–12% PAGE and fluorography. Import was measured by cleavage of the added fusion protein. This cleavage was completely dependent on a mitochondrial membrane potential (not shown here; see Fig. 4). *std*, 10% of the radiolabeled precursor added to each sample. *Mt*, mitochondria; *Mp*, mitoplasts; *OM*, outer membrane.

Results

Disruption of the Outer Membrane Restores Protein Import to Mitochondria Pretreated with Antibody against the Outer Membrane

Earlier experiments in this laboratory had shown that disruption of the outer membrane restored protein import to mitochondria that had been pretreated with high levels of trypsin (Ohba and Schatz, 1987*a*). Since trypsin can stick tightly to membranes (Rietveld et al., 1986) it seemed desirable to verify this restoration effect by an independent procedure. To do this, we inhibited import into mitochondria by IgGs directed against the outer membrane. These IgGs strongly inhibit import into intact mitochondria (Ohba and Schatz, 1987*b*) without the potential nonspecific or destructive side effects of trypsin.

As shown in Fig. 1, mitochondria treated with preimmune IgGs actively imported the radiolabeled pCOXIV-DHFR fusion protein (lane *4*); the efficiency of import (~15% of the added precursor) was essentially the same as that of untreated mitochondria (cf. Ohba and Schatz, 1987*b*). In contrast, IgGs against the outer membrane inhibited import by >80% (lane *2*). This inhibition was almost completely reversed by converting the IgG-pretreated mitochondria to mitoplasts (lane *3*). Pretreatment of mitochondria with nonimmune IgGs before mitoplast preparation did not block import (lane *5*), but resulted in a slight enhancement of import.

Our procedure for breaking the outer membrane released ~90% of the mitochondrial cytochrome b_2 (a soluble enzyme of the intermembrane space), but essentially none of citrate synthase (a soluble enzyme of the matrix) (Fig. 2). Thus, 90% of the mitochondria were converted to mitoplasts.

Disruption of the Outer Membrane also Restores Protein Import to Mitochondria Whose Import Sites Have Been "Jammed" with a Chimeric Protein

Treatment of mitochondria with trypsin or antibodies should inactivate only proteinaceous translocation components (receptors?) that are exposed on the mitochondrial surface. To show that disruption of the outer membrane uncovered novel import sites, we generated mitoplasts from mitochondria whose import sites had been specifically blocked with a chimeric precursor protein. This protein was constructed by covalently crosslinking BPTI to the COOH terminus of a modified pCOXIV-DHFR fusion protein (pDV12; Vestweber and Schatz, 1988). When this chimeric precursor (pDV12-BPTI) is incubated with energized mitochondria, it becomes stuck across both mitochondrial membranes: its NH_2-terminal presequence is cleaved by the matrix-localized protease, its DHFR moiety is inside the mitochondria, and its

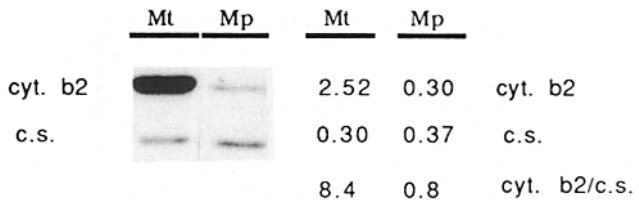

Figure 2. Hypotonic shock efficiently ruptures the mitochondrial outer membrane. Yeast mitochondria were treated with 1 mg trypsin/ml (Materials and Methods) and then converted to mitoplasts by adding 9 vol of 20 mM Hepes-KOH, pH 7.4, 1 mg soybean trypsin inhibitor/ml and incubation for 20 min at 0°C with occasional agitation. The mitoplasts were sedimented by centrifugation at 15,000 *g* for 10 min. (Left) Aliquots (50 μg) of mitochondria (*Mt*) or mitoplasts (*Mp*) were analyzed by SDS–12% PAGE and immune blotting with antisera against cytochrome b_2 (cyt. b_2; intermembrane space marker) and citrate synthase (*c.s.*; matrix marker). (Right) The levels of the two marker proteins in mitochondria and mitoplasts were quantified by densitometric scanning of the autoradiograms and are given in arbitrary units. Conversion to mitoplasts was calculated according to the equation

$$\left[1 - \frac{\text{cyt. } b_2/\text{c.s. } (Mp)}{\text{cyt. } b_2/\text{c.s. } (Mt)} \right] \times 100.$$

In the experiment shown in the figure, conversion efficiency was 90%.

BPTI moiety remains exposed on the mitochondrial surface. This arrangement indicates a location across the contact sites between the two membranes. This view is supported by two independent observations. First, the stuck precursor cofractionates with a submitochondrial membrane fraction whose density is between that of the inner and outer membranes (Pon, L., manuscript in preparation). Second, mitochondria that have accumulated the partly translocated precursor can no longer import authentic precursor proteins even though they can still maintain a membrane potential. Quantitation of this inhibition had indicated that the chimeric precursor titrates a limited number of mitochondrial import sites (Vestweber and Schatz, 1988).

In the experiment documented in Fig. 3, incubation of intact mitochondria with the purified chimeric precursor blocked subsequent import of pCOXIV by ~80% (compare the intensities of the "mature" subunit IV bands, labeled mCOXIV, in lanes *4* and *7*). When these blocked mitochondria were converted to mitoplasts, import of the pCOXIV was restored to a level exceeding even that of the untreated mitochondria (compare the intensities of the mCOXIV bands in lanes *4* and *9*). In all cases, import of pCOXIV was completely prevented by collapsing the membrane potential with valinomycin (lanes *5* and *10*). This experiment also showed that the restored import pathway of mitoplasts operates with an authentic precursor protein. Other experiments extended this observation to the precursors of alcohol dehydrogenase III and the F_1-ATPase β-subunit (not shown). Thus, we conclude that the restored import into mitoplasts uses import sites that are not externally accessible to precursors in the isolated intact mitochondria.

The Restored Import into Mitoplasts Has a Similar Presequence Requirement as Import into Intact Mitochondria

To check whether the import restored to mitoplasts was a bona fide import activity, we tested three pCOXIV-DHFR fusion proteins differing in the length of the attached mitochondrial presequence. Hurt et al. (1985) had previously shown that intact mitochondria can import pCOXIV-DHFR fusion proteins containing as few as 12 NH_2-terminal residues of pCOXIV. As shown in Fig. 4, the same is true for the restored import activity of mitoplasts: shortening the presequence from 12 to 9 residues virtually abolishes import. Import activity of the restored mitoplasts, like that of intact mitochondria, required ATP (not shown) and a potential across the inner membrane.

Restored Protein Import in Mitoplasts Is Sensitive to Low Levels of Proteinase K

We had already observed that the restored import differed from the import into intact mitochondria in its insensitivity

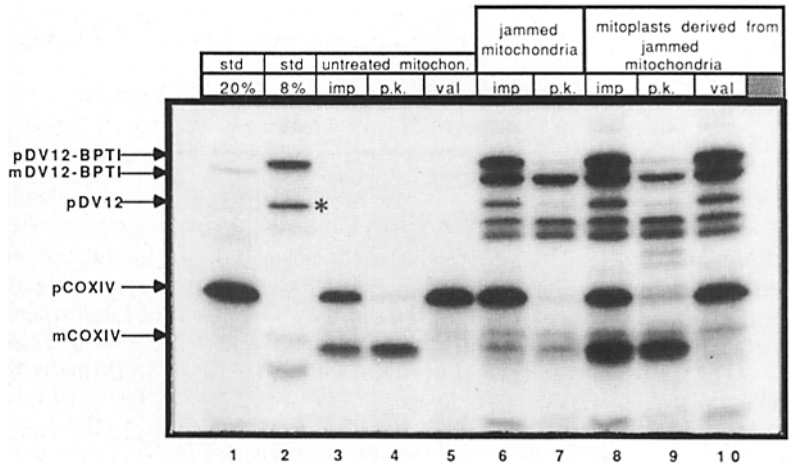

Figure 3. Jamming of import sites in intact mitochondria by a chimeric precursor protein can be bypassed by disrupting the outer membrane. Five aliquots of intact mitochondria (34 μg each) were incubated with the pDV12-BPTI chimeric precursor (562 ng; Vestweber and Schatz, 1988) under ATP-driven import conditions for 20 min at 25°C. Three aliquots of these blocked mitochondria were converted to mitoplasts (see Fig. 2) while two others were maintained under isotonic conditions. After reisolation (at 15,000 *g* for 10 min), these five aliquots, as well as three aliquots of untreated mitochondria (34 μg each), were tested for their ability to import in vitro-synthesized authentic pCOXIV for 10 min at 30°C. Where indicated, valiomycin (*val*; 10 μg/ml) was present during the pCOXIV import assay. After import, some of the samples (as noted) were treated with proteinase K (200 μg/ml; 30 min at 0°C) to digest nonimported proteins. The protease was inhibited and the samples analyzed as in Fig. 1, except that a 13% SDS–polyacrylamide gel was used. *imp*, import conditions; *p.k.*, proteinase K; *mCOXIV*, mature, imported subunit IV; *std*, indicated fraction of the precursor added to each import assay. The band marked by an asterisk is residual underivatized pDV12 protein. As noted before by Vestweber and Schatz (1988), underivatized pDV12 protein is cleaved twice upon import into mitochondria.

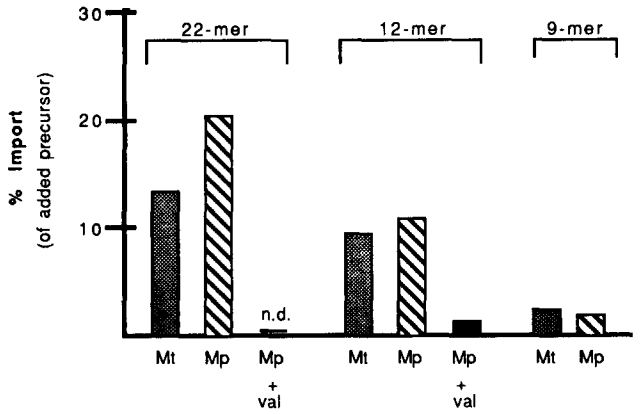

Figure 4. Progressive shortening of a mitochondrial presequence has similar effects on import into mitochondria and mitoplasts. Fusion proteins containing the first 22, 12, or 9 residues of the yeast pCOXIV fused to mouse DHFR were synthesized by coupled transcription/translation in the presence of [^{35}S]methionine and presented to mitochondria or mitoplasts (50 μg protein) under standard ATP-driven import conditions. Mitoplasts had been prepared from mitochondria pretreated for 30 min at 0°C with 1 mg/ml trypsin. Import was for 8 min at 30°C. Where indicated, valinomycin (*val*; 10 μg/ml) was also added. Radiolabeled protein that had become inaccessible to 200 μg proteinase K/ml (30 min at 0°C) was quantified by SDS-12% PAGE, fluorography, and densitometric scanning of the fluorogram. *n.d.*, not detectable; *Mt*, mitochondria; *Mp*, mitoplasts.

to IgGs (or Fab fragments) against the outer membrane or a 45-kD protein of the outer membrane (Ohba and Schatz, 1987*a*). Fig. 5 reveals yet another difference: the restored import is approximately tenfold more sensitive to proteinase K. Even at 15 μg/ml (the highest level tested in Fig. 5), proteinase K did not significantly decrease the ability of the mitoplasts to generate and maintain a membrane potential in the presence of ATP (not shown). Disruption of the mitochondrial outer membrane thus exposes a component of the import machinery that is highly sensitive to proteinase K.

Purified Inner Membrane Vesicles Can Import Precursor Proteins

The results described in the preceding sections strongly suggest that the mitochondrial inner membrane, if exposed directly to precursor proteins, can translocate these proteins without participation of outer membrane proteins. To prove this directly, we disrupted mitochondria by sonication and purified their inner membranes by density gradient centrifugation. On a protein basis, the purified inner membrane vesicles contained approximately tenfold lower concentrations of two outer membrane proteins (porin and 45-kD protein) and a twofold higher concentration of an inner membrane protein (cytochrome oxidase subunit IV [COXIV]) than intact mitochondria (Table I). Furthermore, the inner membrane vesicles equilibrated at a higher density than the vesicles containing the chimeric precursor stuck across the normal mitochondrial import sites (Pon, L., manuscript in preparation).

The inner membrane vesicles generated a membrane potential in the presence of succinate or ascorbate, provided cytochrome *c* was added as well (Fig. 6). The potential was completely collapsed by 1 μg/ml valinomycin.

The energized inner membrane vesicles imported an artificial or an authentic mitochondrial precursor protein as efficiently as intact mitochondria. Fig. 7 *a* shows this for the authentic pCOXIV. In this experiment, import was allowed to proceed only at low temperature (20°C), for short periods, and with very low amounts of inner membrane vesicles to ensure that the amount of precursor imported was directly proportional to time and amount of vesicles. Import of the pCOXIV into the vesicles was accompanied by partial conversion of the precursor to an intermediate form (intermediate COXIV; see Hurt et al., 1985) and mature form (mature COXIV) and was sensitive to valinomycin. In addition, some of the precursor molecules became inaccessible to externally added protease without being cleaved to smaller forms, presumably because the matrix-localized processing protease had been partly lost or inactivated during preparation of the particles. Fig. 7 *b* shows that the import activity of the vesicles is actually higher than that of an equal amount of mitochondria.

To compare the "specific import activities" of inner membrane vesicles and mitochondria, we related import activity with the amount of an inner membrane protein, COXIV (Table I). Based on this calculation, the inner membrane vesicles imported an authentic precursor (pCOXIV) twice as fast and an artificial precursor (pCOXIV-DHFR) half as fast as mitochondria, even though they had lost ~90% of two outer membrane markers.

Import of the pCOXIV-DHFR protein into the inner membrane vesicles, as into mitochondria, required ATP as well as a membrane potential (not shown). ATP could be replaced by GTP. As with intact mitochondria, nucleoside triphosphates other than ATP may thus be active in driving import (Eilers et al., 1987). Moreover, the import activity of puri-

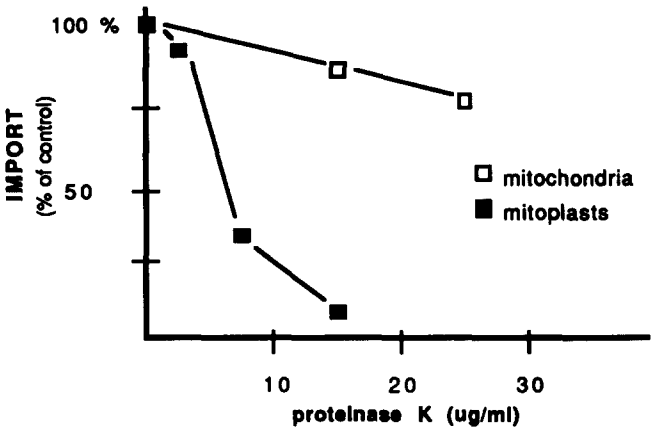

Figure 5. Import into mitoplasts, but not into mitochondria, is sensitive to low levels of proteinase K. Mitochondria and mitoplasts derived from trypsin-pretreated mitochondria were incubated with the indicated concentrations of proteinase K in the presence of soybean trypsin inhibitor (see Materials and Methods) and then allowed to import the radiolabeled pCOXIV-DHFR fusion protein for 7 min at 30°C. Each assay contained 100 μg of particle protein. The amount of cleaved fusion protein (quantified by densitometry) was taken as a measure of import since cleavage was completely blocked by 10 μg valinomycin/ml (not shown). Import values were normalized to import in the absence of preexposure to proteinase K (10% and 15% of added precursor, respectively, for mitoplasts and mitochondria), which was taken as 100%.

Table I. Inner Membrane Vesicles Import Artificial or Authentic Precursor Proteins As Efficiently As Intact Mitochondria

	Mitochondria	Inner membrane vesicles
Porin/μg protein	15.0	1.4
45-kD OM/μg protein	71.0	9.1
COXIV/μg protein	9.0	17.0
Percent import pCOXIV/U COXIV	0.03	0.06
Percent import pCOXIV-DHFR/U COXIV	0.14	0.06

Three different amounts (5, 10, and 50 μg) of inner membrane vesicles and intact mitochondria were analyzed by 13% SDS-PAGE and immune blotting with polyclonal rabbit antisera against porin, 45-kD outer membrane protein (*45-kD OM*), and COXIV. Antigen bands were quantified by autoradiography and scanning densitometry and expressed in arbitrary scanner units/μg of vesicle protein or mitochondrial protein. To determine the specific activity of import, 10 μg of mitochondria or inner membrane vesicles (the same preparations as used in the antigen quantification) were allowed to import 150 ng of purified pCOXIV-DHFR in the presence of respiratory substrates and ATP for 8 min at 30°C (see Fig. 7 *a*) and then treated with proteinase K (200 μg/ml, 30 min at 0°C). The samples were analyzed by 12% SDS-PAGE, fluorography, and densitometric scanning. In a separate experiment with another preparation of vesicles and mitochondria (see Fig. 7, *a* and *b*), import of in vitro-synthesized authentic pCOXIV was tested for 7 min at 20°C and analyzed by 13% SDS-PAGE, fluorography, and densitometry scanning. Specific activity in both import experiments is expressed as percent of added precursor imported per arbitrary scanner unit of COXIV, an inner membrane marker. The inner membrane vesicles possessed 1.88-fold more COXIV per μg protein than intact mitochondria.

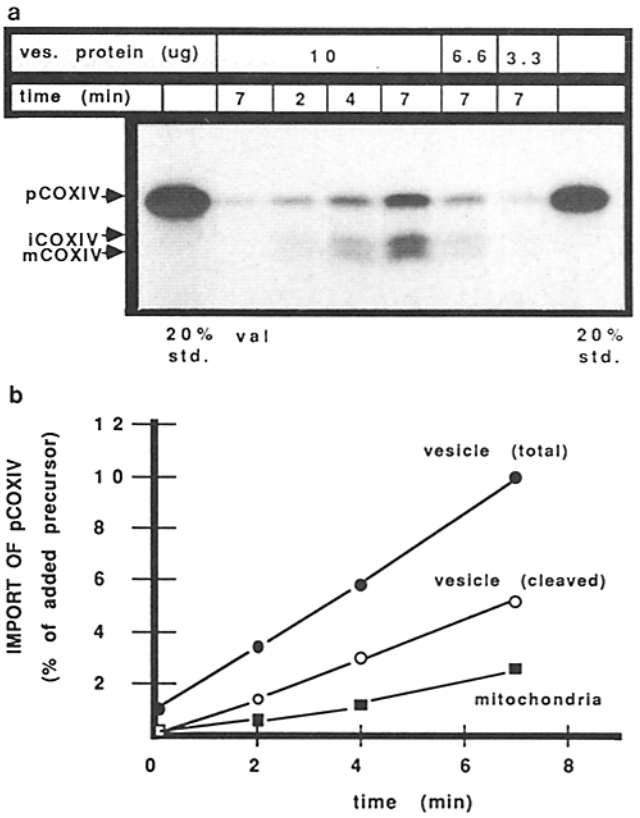

Figure 7. Purified inner membrane vesicles import pCOXIV as efficiently as intact mitochondria. (*a*) Inner membrane vesicles (3.3, 6.6, or 10 μg in import buffer supplemented with 10 mM succinate, 10 mM malate, 3 mM ascorbate, and 0.5 mg cytochrome *c*/ml, final volume 200 μl) were mixed with 10 μl of in vitro-synthesized pCOXIV. After the indicated incubation times at 20°C, import was inhibited by valinomycin (10 μg/ml), and the samples were treated with proteinase K (100 μg/ml final for 30 min on ice) to digest nonimported protein and then sedimented (Beckman Airfuge; Beckman Instruments, Inc., Fullerton, CA; at 130,000 *g* for 20 min at 4°C). Valinomycin (lane *val*; 10 μg/ml final) was added to one sample before the addition of the precursor to demonstrate the membrane potential dependence of import. *20% std.*, 20% of the precursor added to each sample. *iCOXIV*, intermediate cleavage form of imported subunit IV; *mCOXIV*, mature form of imported protein. (*b*) A similar import experiment was carried out with mitochondria using the same amounts of protein and import times at 20°C as for the vesicles. For mitochondria, import is quantified as the amount of protease-resistant mature COXIV. For vesicles, import (*a*) is expressed either as the sum of protease-resistant precursor form, cleaved intermediate and mature form (*total*) or as the sum of the two cleaved forms (*cleaved*). Samples were analyzed by SDS-13% PAGE, fluorography, and densitometry of the fluorograms.

fied inner membrane vesicles, unlike that of intact mitochondria, was insensitive to IgGs against the outer membrane (Fig. 8). This makes it unlikely that osmotic shock restores import to antibody-treated mitochondria (Fig. 1) by displacing bound IgGs. The combined results strongly indicate that import into the inner membrane vesicles is not mediated by residual outer membrane components, but reflects an intrinsic activity of the inner membrane itself.

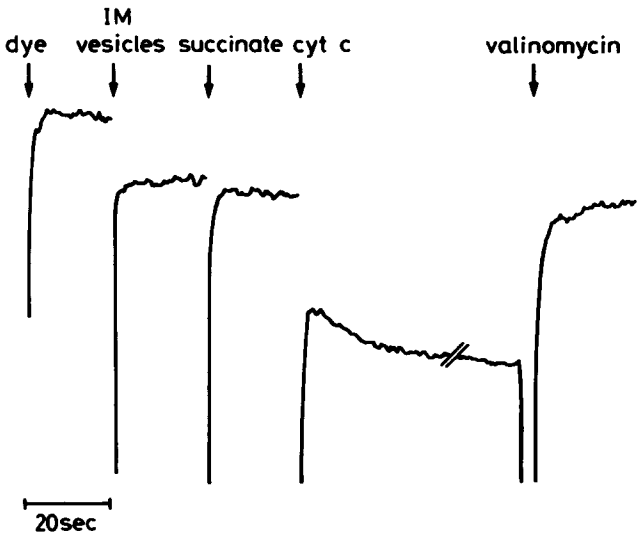

Figure 6. Inner membrane vesicles can generate a membrane potential. The potential was measured fluorometrically with the potential-sensitive dye 3,3′dipropylthiocarbocyanine iodide in the presence of 20–30 μg of inner membrane (*IM*) vesicle protein in a total volume of 2.5 ml (see Materials and Methods). Where indicated, dye was added to 4 μM, succinate to 5 mM, horse heart cytochrome *c* (*cyt. c*) to 50 μg/ml, and valinomycin to 1 μg/ml. Fluorescence is given in arbitrary units; an increase in potential is represented by a downward deflection. The break in the fluorescence measurement represents 1.5 min.

Discussion

The data presented in this paper lead us to conclude that the mitochondrial inner membrane can translocate mitochondrial precursor proteins even without participation of outer

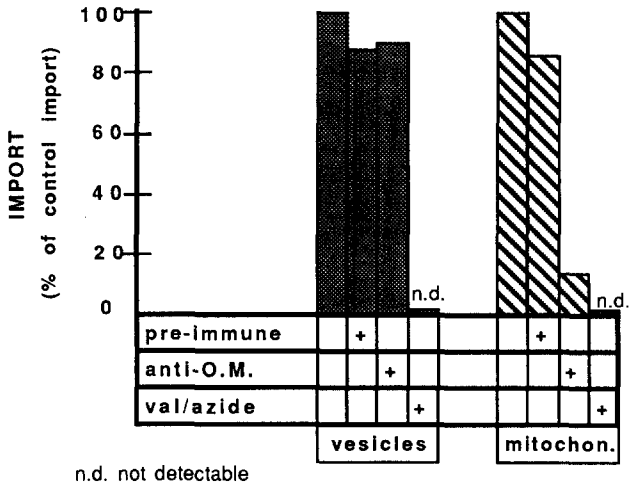

Figure 8. Import of the pCOXIV-DHFR fusion protein into inner membrane vesicles is not inhibited by anti-outer membrane IgGs. 10 μg of inner membrane vesicles or mitochondria were preincubated in 77 μl with 500 μg of either anti-outer membrane IgGs (*anti-O.M.*) or preimmune IgGs in 0.6 M sorbitol, 20 mM KPi, pH 7.4, on ice for 1 h. The suspension was then mixed with 85 μl of import buffer and supplemented with 10 mM succinate, 10 mM malate, 3 mM ascorbate, 0.5 mg cytochrome *c*/ml, ATP plus an ATP-regenerating system, and 150 ng (10 μl) of purified ^{35}S-labeled pCOXIV-DHFR fusion protein. After incubation at 30°C for 10 min, import was stopped by the addition of valinomycin (10 μg/ml) and putting the mixture on ice. All samples were treated with proteinase K (200 μg/ml for 30 min on ice), reisolated, and analyzed by 12% SDS-PAGE, fluorography, and densitometric scanning. Values were normalized to import in the absence of antibodies for vesicles and mitochondria which was taken as 100%. The absolute values for import into mitochondria and vesicles were 6.6 and 5%, respectively, of added precursor. Based on the amount of the inner membrane marker, COXIV, the antibody-resistant import activity of the vesicles was more than twofold higher than the residual import activity of the antibody-treated mitochondria. *val/azide*, mitochondria or vesicles pretreated with valinomycin (10 μg/ml) and sodium azide (4 mM) before import.

membranes. This translocation activity resembles that of intact mitochondria in several respects: it requires a potential across the inner membrane; it depends on a minimal length of the "matrix-targeting" sequence; and it requires ATP. On the other hand, it differs from that of mitochondria in being highly sensitive to proteinase K and insensitive to antibody against outer membrane components.

The experiments with purified inner membrane vesicles are the first demonstration of protein import activity in a submitochondrial system. They also provide particularly compelling evidence that inner membranes by themselves can translocate mitochondrial precursors if they are allowed direct access to them.

It might be argued that osmotic shock reactivates import to the blocked mitochondria by displacing the blocking agents (antibodies or chimeric precursor). However, restoration by osmotic shock is observed after three rather different blocking procedures: protease-treatment (Ohba and Schatz, 1987*a*), incubation with antibody against outer membrane, and jamming with a chimeric precursor protein. While each of these procedures might be questioned independently, together they make a rather persuasive case.

How valid is the evidence that import into inner membrane vesicles is not caused by residual outer membranes or contact sites? While it is always difficult to prove that something is absent, we feel that the involvement of outer membrane components is very unlikely: compared to intact mitochondria, the inner membrane vesicles have lost ~90% of two characteristic outer membrane proteins, yet import is comparable to that of intact mitochondria. It is more difficult to exclude the presence of residual contact sites since a reliable specific marker protein for this structure is not yet available. However, when most of the import sites of intact mitochondria are blocked by a chimeric protein which, by all criteria tested, appears to become stuck across contact sites, disruption of the outer membrane completely restores import. The restored import thus bypasses at least those import sites which are active in intact mitochondria.

Our data are compatible with, but do not prove, the notion that import-competent contact sites are dynamic structures that may be formed in response to a specific signal, such as the binding of a precursor to the mitochondrial surface. Indeed, electron microscopic studies of mammalian mitochondria have led Ohlendieck et al. (1986) to conclude that contacts between the two mitochondrial membranes vary depending on the physiological state of the mitochondria.

In a living cell, precursors destined for the matrix space must be specifically targeted to mitochondria and penetrate across both mitochondrial membranes. It is now clear that this process requires proteins of the outer membrane (Riezman et al., 1983*b*; Zwizinski et al., 1983, 1984; Ohba and Schatz, 1987*a*,*b*); our data raise the possibility that these proteins may bind precursors, partly change their conformation (Eilers et al., 1988, 1989; Endo et al., 1989), and transport them across the outer membrane, but may not be mechanistically involved in transporting them across the inner membrane.

The inner membrane vesicles described here are a particularly simple system for studying protein import into mitochondria. They should be useful for identifying and isolating components of the mitochondrial translocation machinery.

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