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

S. Hwang, T. Jascur, D. Vestweber, L. Pon, and G. Schatz

Biocenter, Department of Biochemistry, University of Basel, CH-4056 Basel, Switzerland

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.

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) 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 MgCl2. 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 (1987a). Trypsin-treated mitochondria, mitoplasts, and untreated mitochondria were suspended to 5 mg protein/ml in import buffer. 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 (~143 ml) were disrupted by sonication (macrotip; Heat Systems-Ultrasoundics, 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 densities 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 MgCl₂, 0.5 mM EDTA, 20 mM KPi, pH 7.4, 0.6 M sorbitol, and 1 mg/ml BSA. A 2-µM 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., 1983a,b; Ohba and Schatz, 1987b). 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).

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 into mitochondria that had been pretreated with high levels of trypsin (Ohba and Schatz, 1987a). 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, 1987b) 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, 1987b). 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 non-immune 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 b₁ (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 NH₂-terminal presequence is cleaved by the matrix-localized protease, its DHFR moiety is inside the mitochondria, and its
Hwang et al. Disrupted Yeast Mitochondria

489

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 b2 (cyt. b2; 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

\[
\left[ 1 - \frac{\text{cyt. b2/c.s. (Mp)}}{\text{cyt. b2/c.s. (Mt)}} \right] \times 100.
\]

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 F1-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 NH2-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 (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.
to IgGs (or Fab fragments) against the outer membrane or port is approximately tenfold more sensitive to proteinase K. A 45-kD protein of the outer membrane (Ohba and Schatz, 1987) contained approximately tenfold lower concentrations directly to precursor proteins, can translocate these 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 sonicaton 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 1). 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-
Table 1. 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.

Fried 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.

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
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 sub-mitochondrial 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, 1987a), 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 Ohlendiek 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., 1983b; Zwizinski et al., 1983, 1984; Ohba and Schatz, 1987a,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.

We wish to thank Dr. Masayuki Ohba for kind support; Dr. Toshiya Endo and Dr. Martin Eilers for advice as well as for purified pCOXIV-DHFR; and Michele Probst for secretarial assistance.

This study was supported by grant 3.335.0.86 from the Swiss National Science Foundation and grant CBY-11 R01 GM37803-01 from the U.S. Public Health Service. Sam Hwang was a recipient of the Stanley Sarnoff Fellowship for Cardiovascular Science. L. Pen is a recipient of the National Research Service Award (5 F32 GM11799-02 BI-3) from the National Institute of General Medical Sciences.

Received for publication 3 March 1989 and in revised form 17 April 1989.

References

Attaardi, G., and G. Schatz. 1988. The biogenesis of mitochondria. Annu. Rev. Cell Biol. 4:289-333.

Daum, G., P. C. Boehni, and G. Schatz. 1982. Import of proteins into mitochondria: cytochrome b₄ and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. J. Biol. Chem. 257:13028-13033.

Eilers, M., and G. Schatz. 1986. Binding of specific ligand inhibits import of a purified precursor protein into mitochondria. Nature (Lond.). 322:228-232.

Eilers, M., W. Oppliger, and G. Schatz. 1987. Both ATP and an energized inner membrane are required to import a purified precursor protein into mitochondria. EMBO (Eur. Mol. Biol. Organ.) J. 6:1073-1077.

Eilers, M., S. Hwang, and G. Schatz. 1988. Unfolding and refolding of a purified precursor protein during import into isolated mitochondria. EMBO (Eur. Mol. Biol. Organ.) J. 7:1139-1145.
Eilers, M., T. Endo, and G. Schatz. 1989. Adriamycin, a drug interacting with acidic phospholipids, blocks import of precursor proteins by isolated yeast mitochondria. *J. Biol. Chem.* 264:2943–2950.

Endo, T., M. Eilers, and G. Schatz. 1989. Binding of a tightly folded artificial mitochondrial precursor protein to the mitochondrial outer membrane involves a lipid-mediated conformational change. *J. Biol. Chem.* 264:2951–2956.

Hackenbrock, C. R. 1968. Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states. *Proc. Natl. Acad. Sci. USA.* 61:598–605.

Hurt, E. C., B. Pesold-Hurt, and G. Schatz. 1984. The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3149–3156.

Hurt, E. C., B. Pesold-Hurt, K. Soda, W. Oppliger, and G. Schatz. 1985. The first twelve amino acids (less than half of the pre-sequence) of an imported mitochondrial protein can direct mouse cytosolic dihydrofolate reductase into the yeast mitochondrial matrix. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2061–2068.

Kelles, R. E., V. F. Allison, and R. A. Butow. 1975. Cytoplasmic type 80S ribosomes associated with yeast mitochondria. IV. Attachment of ribosomes to the outer membrane of isolated mitochondria. *J. Cell Biol.* 65:1–14.

Ohba, M., and G. Schatz. 1987a. Disruption of the outer membrane restores protein import to trypsin-treated yeast mitochondria. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2117–2122.

Ohba, M., and G. Schatz. 1987b. Protein import into yeast mitochondria is inhibited by antibodies raised against 45-kD proteins of the outer membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2109–2115.

Ohlendieck, K., I. Riesinger, V. Adams, J. Krause, and D. Brdiczka. 1986. Enrichment and biochemical characterization of boundary membrane contact sites from rat-liver mitochondria. *Biochim. Biophys. Acta.* 860:572–589.

Pain, D., Y. S. Kanwar, and G. Blobel. 1988. Identification of a receptor for protein import into chloroplasts and its localization to envelope contact zones. *Nature (Lond.)*. 331:232–236.

Pluemer, N., and W. Neupert. 1986. Transport of F1-ATPase subunit beta into mitochondria depends on both a membrane potential and nucleoside triphosphates. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 209:152–156.

Rietveld, A., W. Jordi, and B. de Kruijff. 1986. Studies on the lipid dependency and mechanism of the translocation of the mitochondrial precursor protein apocytochrome c across model membranes. *J. Biol. Chem.* 261:3846–3856.

Riezman, H., R. Hay, S. Gasser, G. Daum, G. Schneider, C. Witte, and G. Schatz. 1983a. The outer membrane of yeast mitochondria: isolation of outside-out sealed vesicles. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1105–1111.

Riezman, H., R. Hay, C. Witte, N. Nelson, and G. Schatz. 1983b. Yeast mitochondrial outer membrane specifically binds cytoplasmically-synthesized precursors of mitochondrial proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1113–1118.

Schwaiger, M., V. Herzog, and W. Neupert. 1987. Characterization of translocation contact sites involved in the import of mitochondrial proteins. *J. Cell Biol.* 105:235–246.

Sims, P. J., A. S. Waggoner, C.-H. Wang, and J. F. Hoffman. 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. *Biochemistry.* 13:3315–3330.

Vestweber, D., and G. Schatz. 1988. A chimeric mitochondrial precursor protein with internal disulfide bridges blocks import of authentic precursors into mitochondria and allows quantitation of import sites. *J. Cell Biol.* 107:2037–2043.

Zwizinski, C., M. Schleyer, and W. Neupert. 1983. Transfer of proteins into mitochondria: precursor to the ADP/ATP carrier binds to receptor sites on isolated mitochondria. *J. Biol. Chem.* 258:4071–4074.

Zwizinski, C., M. Schleyer, and W. Neupert. 1984. Proteinaceous receptors for the import of mitochondrial precursor proteins. *J. Biol. Chem.* 259:7850–7856.