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Minireview

Coupling Chemical Energy by the hsp70/tim44 Complex to Drive Protein Translocation into Mitochondria[1]

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A dynamic complex between the mitochondrial cognate of hsp70 (mthsp70) and the inner membrane protein tim44 couples energy derived from ATP hydrolysis to drive multiple steps in the mitochondrial protein import pathway: (1) The $\Delta\Psi$ dependent import step and the mthsp70/tim44 complex cooperate to facilitate the unidirectional transfer of the mitochondrial targeting signal across the inner membrane. (2) The mthsp70/tim44 complex helps to unfold domains on precursors proteins that arrive at the import apparatus in a folded conformation on the *cis* side of the outer membrane. (3) Completion of import is then driven by the mthsp70/tim44 complex in a manner that is independent of $\Delta\Psi$. Mechanisms proposed to explain how the mthsp70/tim44 complex harvests chemical energy to drive these aspects of the import process are discussed.

KEY WORDS: Mitochondrial biogenesis; protein translocation; hsp70; DnaJ proteins; molecular chaperones.

INTRODUCTION

How the cell solves the problem of transporting hydrophilic polypeptides across the membranes that enclose subcellular organelles is a central question in biology (Blobel, 1980). Study of the pathway for protein translocation into mitochondria has provided paradigms for general mechanisms by which proteins are translocated across different membrane systems (Glick, 1995; Pfanner and Meijer, 1995; Schatz and Dobberstein, 1996; Stuart *et al.*, 1994a; Wickner, 1994). Protein translocation into mitochondria is a complex multi-step process that requires signals for the targeting of proteins synthesized on cytosolic ribosomes to the outer membrane (reviewed by David Roise in this series). To enter the import apparatus proteins must assume an unfolded conformation (Eilers and Schatz, 1986). Targeting signals must also be exposed so that they can be recognized by specific soluble and/or membrane bound import receptor complexes (reviewed by Trevor Lithgow in this series). Once bound to the outer membrane import machinery, precursor proteins are transferred through a protein and possibly lipid-lined channel that permits passage of polypeptides into the intermembrane space. Once in the intermembrane space the presequence is recognized by an independent import machinery in the inner membrane and is transferred through to a second protein translocation channel in a $\Delta\Psi$ dependent import step (reviewed by Rob Jensen in this series). Upon entry into the matrix the presequence is bound by mthsp70/tim44 complex which facilitates the completion of the import process (Cyr *et al.*, 1993; Stuart *et al.*, 1994a; Glick *et al.*, 1993; Pfanner and Meijer, 1995). Finally, newly imported polypeptides are folded and assembled into active molecules in reactions that often require the action of molecular chaperone proteins (reviewed by Jorg Martin in this series).

How the mthsp70/tim44 complex utilizes energy derived from ATP hydrolysis to drive protein translocation into mitochondria is the subject of debate (Glick 1995; Pfanner and Meijer 1995; Stuart *et al.*, 1994a). In this review a brief overview of the energetics of

[1] Abbreviations: hsp70, 70 kilodalton heat shock protein.

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protein translocation into mitochondria will be provided and then recent experiments which refine steps in the $\Delta\Psi$ and mthsp70/tim44 dependent reaction cycle that drives the import process will be discussed. Aspects of the molecular ratchet and motor models proposed to explain how the mthsp70/tim44 complex drives unidirectional transfer of proteins into mitochondria will then be presented.

ENERGY REQUIREMENTS FOR PROTEIN TRANSLOCATION INTO MITOCHONDRIA

The process of protein translocation into mitochondria is fueled by the energy derived from ATP hydrolysis and the membrane potential across the inner membrane ($\Delta\Psi$). $\Delta\Psi$ is essential for translocation of the presequence into the matrix (Schleyer and Neupert, 1985). In the absence of $\Delta\Psi$, the presequence binds mitochondria and is translocated across the outer membrane, but it cannot move beyond the intermembrane space (Mayer *et al.*, 1995). The exact mechanism of how $\Delta\Psi$ is consumed to drive the import of the presequence is unknown, but an electrophoretic mechanism has been proposed (Pfanner and Meijer, 1995). Once the presequence has been transferred deep into the matrix and has been proteolytically processed, ATP-dependent completion of import occurs independent of $\Delta\Psi$ (Schleyer and Neupert, 1985).

Import reactions that require ATP occur both in the cytosol and mitochondrial matrix (Chen and Douglas 1987; Hwang and Schatz, 1989). Cytosolic hsp70/DnaJ chaperone pairs bind and release precursor proteins in an ATP-dependent cycle to maintain them in transport-competent conformations prior to interaction with the import machinery (Gething and Sambrook, 1992; Cyr *et al.*, 1994). Another cytosolic protein complex involved in the import pathway is the mitochondrial import stimulating factor (MSF; Hachiya *et al.*, 1995). MSF consumes ATP to interact with precursor proteins in a presequence-dependent manner and helps target them to interact with subcomponents of the import receptor complex in the outer membrane (Hachiya *et al.*, 1995). Although important for the overall import process, cytosolic ATP is not essential to drive the actual protein translocation reaction (Neupert *et al.*, 1990). If preproteins arrive at the import machinery in a loosely folded conformation they enter the matrix without the energy derived from hydrolysis of cytosolic ATP (Hwang and Schatz, 1989; Stuart *et al.*, 1994a). Energy derived from ATP hydrolyzed in the matrix by mthsp70 is on the other hand essential to drive the import reaction (Hwang and Schatz, 1989). The sections below highlight data which demonstrates that mthsp70 functions in conjunction with tim44 to catalyze several specific steps in the mitochondrial import pathway.

THE mthsp70/tim44 COMPLEX IS ESSENTIAL FOR PROTEIN TRANSLOCATION

To facilitate the import process mthsp70 must form a complex with tim44 (Kronidou *et al.*, 1994; Schneider *et al.*, 1994; Rassow *et al.*, 1994). Tim44 is a peripheral inner membrane protein that is a component of the inner membrane protein translocation machinery (Maarse *et al.*, 1992; Berthold *et al.*, 1995). Tim44 forms a dynamic 1:1 complex with mthsp70 that is sensitive to adenine nucleotides (Kronidou *et al.*, 1994; Schneider *et al.*, 1994; Rassow *et al.*, 1994). Tim44 appears to have roles in the import process that are analogous to the functions of the DnaJ family of co-chaperone proteins (Cyr *et al.*, 1994). Tim44 concentrates mthsp70 to the import site and puts it in position to interact with polypeptides simultaneous with their entry into the matrix. Tim44 can be cross-linked to and co-immunoprecipitated with protein translocation intermediates and therefore appears to be a polypeptide binding protein (Kronidou *et al.*, 1994; Schneider *et al.*, 1994; Rassow *et al.*, 1994; Ungermann *et al.*, 1996). Tim44 might bind and then transfer segments of incoming chains to mthsp70 during the import process. How interactions between mthsp70, tim44, and the incoming chains are regulated by adenine nucleotides is currently under study.

THE mthsp70/tim44 COMPLEX FACILITATES MULTIPLE ASPECTS OF PROTEIN TRANSLOCATION ACROSS THE INNER MEMBRANE OF MITOCHONDRIA

Since the initial biochemical (Scherer *et al.*, 1990) and genetic (Kang *et al.*, 1990) studies which established the requirement for mthsp70 in the import process, its role has been refined continuously. Current data suggests that the mthsp70/tim44 complex facilitates at least three distinct types of reactions that are essential for the import process to occur. (i) Mthsp70/tim44 interacts with mitochondrial targeting sequences

upon their exposure to the matrix and thereby stabilizes them on the *trans* side of the inner membrane. This important mthsp70 action serves to make the initial import step irreversible and represents the first step of commitment for the precursor in the import process (Cyr *et al.*, 1993; Ungermann *et al.*, 1994, 1996). (ii) Mthsp70, by binding to matrix-exposed parts of preproteins, serves to secure the unfolding of tightly-folded segments of preproteins on the *cis* side of the outer membrane (Stuart *et al.*, 1994b; Glick *et al.*, 1993; Voos *et al.*, 1993). (iii) Through a series of binding and release cycles, to additional domains of the preprotein, mthsp70 action drives the completion of translocation across the inner membrane (Neupert *et al.*, 1990; Scherer *et al.*, 1990). Details of the different aspects of mthsp70 function in these subreactions of the mitochondrial protein import pathway are provided below.

UNIDIRECTIONAL TRANSFER OF THE PRESEQUENCE ACROSS THE INNER MEMBRANE REQUIRES BOTH $\Delta\Psi$ AND mthsp70/tim44

The mitochondrial presequence is transferred across the inner membrane in a $\Delta\Psi$ dependent reaction (Schleyer and Neupert, 1985). Recent reports that analyze protein translocation intermediates that accumulate when matrix ATP is depleted reveal that in addition to $\Delta\Psi$, the mthsp70/tim44 complex also acts in the process of presequence insertion across the inner membrane (Cyr *et al.*, 1993; Glick *et al.*, 1993; Hwang and Schatz, 1989; Ungermann *et al.*, 1994, 1996). Interference with formation of the mthsp70/tim44 complex causes the presequence of import intermediates to diffuse out of the inner membrane import channel in the presence of high $\Delta\Psi$ (Ungermann *et al.*, 1994, 1996). The presequence apparently oscillates in the import channel during translocation and the mthsp70/tim44 complex serves to trap it on the *trans* side of the inner membrane (Cyr *et al.*, 1993; Ungermann *et al.*, 1994, 1996). $\Delta\Psi$ alone appears insufficient to block reverse translocation of the presequence out of the matrix (Ungermann *et al.*, 1994, 1996). The $\Delta\Psi$ and mthsp70/tim44 dependent import machinery therefore must cooperate to lock incoming chains in the import channel and confer unidirectionality on the initial steps in protein translocation across the inner mitochondrial membrane.

IN SOME CASES mthsp70/tim44 FACILITATES THE UNFOLDING OF PRECURSORS OUTSIDE THE MITOCHONDRIA

Precursor proteins cannot cross membranes in a folded conformation (Eilers and Schatz 1986). During import it appears that segments of 50 amino acid residues of incoming precursor proteins span the import channels in an extended conformation (Rassow *et al.*, 1990). These constraints require that precursor proteins destined for the matrix do not fold tightly upon translation in the cytosol. Folding of precursor proteins is prevented by a number of factors in the cytosol. These include hindrance of folding by the targeting signal or presequence, the lack of bound prosthetic groups which sometimes form an integral part of the mature functional enzyme, and, as discussed previously, interaction of cytosolic chaperones with precursors. On the other hand, it is clear that subdomains of some preproteins do indeed fold tightly while in the cytoplasm and yet these preproteins are imported efficiently into mitochondria (Glick *et al.*, 1993). How does the import machinery cope with such folded domains? Does a mechanism exist to "unfold" domains on precursor proteins after they engage the import machinery? The answer to this question is yes and the mthsp70/tim44 complex plays an important role in the process of preprotein unfolding.

During the course of studying the energy requirements of the import of the precursor of cytochrome b_2 (pb_2), it was demonstrated that mthsp70 activity is required for the unfolding of tightly-folded segments in the mature part of this precursor outside of mitochondria. Cytochrome b_2 (L-lactate dehydrogenase) is located in the intermembrane space and contains both heme and flavin as prosthetic groups. The initial 100 amino acid residues of the mature cytochrome b_2 polypeptide chain constitute a tightly-folded structure, termed the cytochrome b_5 or heme binding domain (Xia and Matthews, 1990). The heme binding domain folds in the cytosol prior to its translocation across the outer mitochondrial membrane (Glick *et al.*, 1993).

The import of cytochrome b_2 displayed a very strong requirement for matrix ATP (Glick *et al.*, 1993; Stuart *et al.*, 1994b). In the absence of matrix ATP, the precursor accumulated as an unprocessed species on the outer surface of mitochondria. Similar results were obtained if import was performed using mitochondria prepared from mutant strains of yeast with defects in mthsp70 (Voos *et al.*, 1993). The necessity

for matrix ATP for cytochrome b_2 import reflected a dependence on mthsp70. Furthermore, if the precursor of cytochrome b_2 was unfolded in 8 M urea prior to import, it could be imported very efficiently into both ATP-depleted mitochondria and into mthsp70 mutant mitochondria (Glick *et al.*, 1993; Stuart *et al.*, 1994b; Voos *et al.*, 1993).

If subdomains on precursor proteins fold into stable structures prior to entering the import channel, this can prevent further import of the polypeptide when the folded domain comes in contact with the outer membrane. ATP-dependent binding of mthsp70 to matrix exposed parts can facilitate the unfolding of portions of import intermediates that are outside of mitochondria. How mthsp70 overcomes the thermodynamic barriers that stabilize folded subdomains on polypeptides is a matter of debate (Glick, 1995; Stuart *et al.*, 1994a). The molecular ratchet model predicts that mthsp70 uses energy derived from ATP hydrolysis to unfold import intermediates by binding them and shifting the equilibrium of spontaneous unfolding reactions away from the native state (Stuart *et al.*, 1994a). The molecular motor model predicts that ATP hydrolysis powers a conformational change in mthsp70 that generates a force which pulls folded domains on preproteins apart (Glick, 1995).

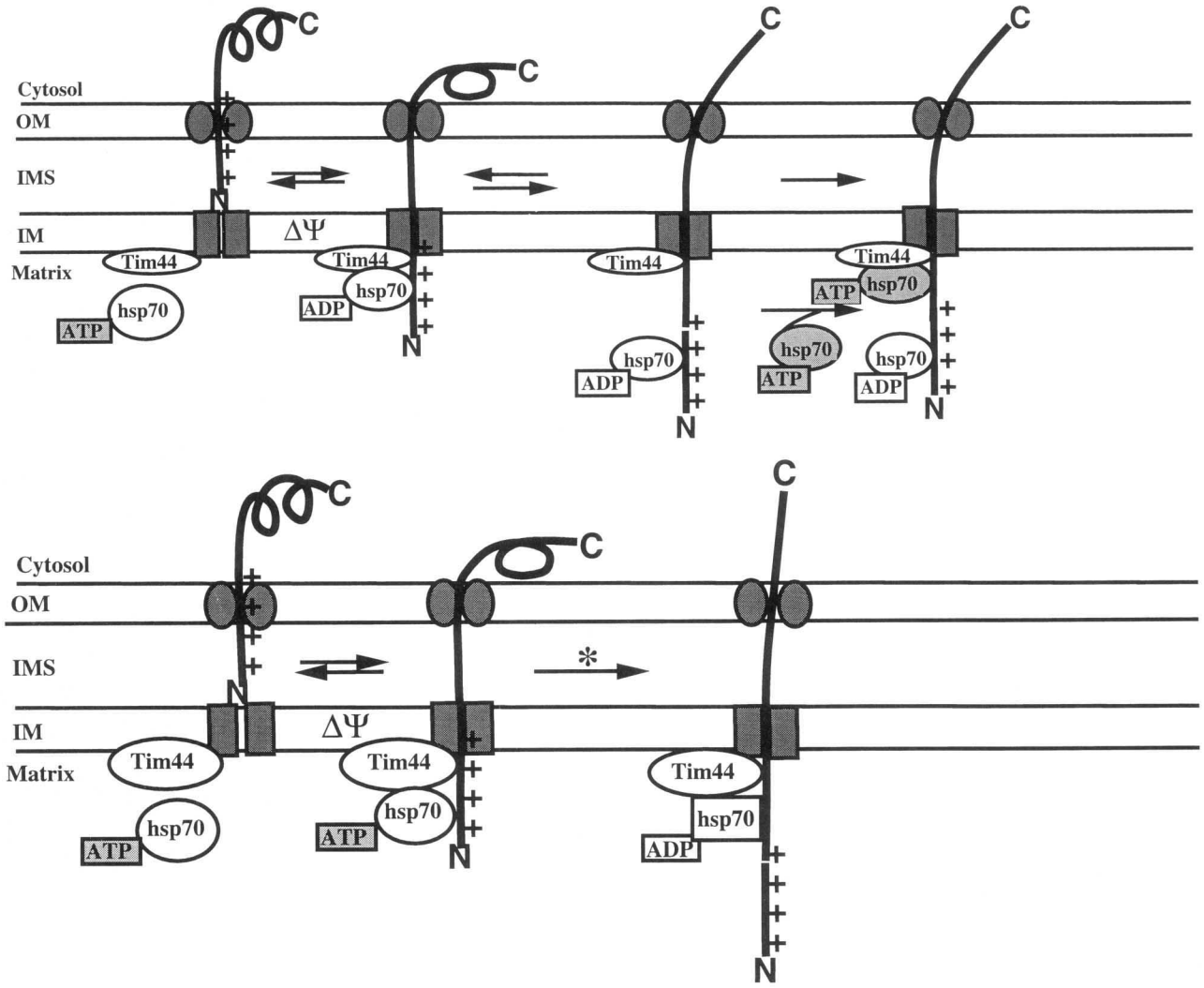

Fig. 1. Proposed models of how the mthsp70/tim44 complex drives protein translocation into mitochondria. The shaded ovals and squares denote the inner (IM) and outer (OM) membrane protein translocation machineries. Top: the molecular ratchet model. Bottom: the molecular motor model. The plus signs (+) mark regions of the mitochondrial presequence. Processing of the presequence is not denoted, but appears to occur after release of mthsp70 from the incoming chain.

COMPLETION OF IMPORT IS DRIVEN BY THE mthsp70/tim44 COMPLEX

Mthsp70 and tim44 cooperate to drive polypeptides that are inserted across the inner membrane into the matrix. This process occurs in a reaction cycle that involves the interaction of the incoming chain with both mthsp70 and tim44 (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994; Ungermann *et al.*, 1994, 1996). There is evidence to support the proposal that the mthsp70 and tim44 function as a molecular ratchet to drive the import process (Fig. 1A; Berthold *et al.*, 1995; Schneider *et al.*, 1994; Simon *et al.*, 1992; Ungermann *et al.*, 1994, 1996). There are also alternative arguments for the mthsp70/tim44 complex acting as molecular motor to pull polypeptides into the matrix (Fig. 1B; Glick *et al.*, 1993; Glick, 1995; Pfanner and Meijer, 1995). In both models incoming chains are delivered to mthsp70 that is localized to the matrix face of the inner membrane through complex formation with tim44. It appears that the ATP form of mthsp70 binds tim44 and the incoming chain in a reaction that is accompanied by the hydrolysis of ATP. At this point the different models for hsp70 action diverge. In the motor model the conformational change in mthsp70 that occurs upon ATP hydrolysis is proposed to generate a force that is sufficient to pull polypeptides across the inner membrane (Glick, 1995). The ratchet model predicts that ATP hydrolysis destabilizes the complex between mthsp70 and tim44, while stabilizing the interaction of this chaperone with the incoming chain (Schneider *et al.*, 1994; Ungermann *et al.*, 1996). This causes the mthsp70/precursor protein complex to dissociate from tim44, which unlocks the incoming chain from the import channel and allows it to diffuse reversibly in the membrane. Diffusion of import intermediates out of the inner membrane is limited by the presence of mthsp70 on the incoming chain. Deeper movement of incoming chains into the matrix then proceeds via brownian motion (Neupert *et al.*, 1990; Simon *et al.*, 1992). When the incoming chain has moved deep enough it is bound again by the mthsp70/tim44 complex. This reaction locks the chain in the import apparatus and confers net movement on the translocation reaction cycle. Release of ADP from mthsp70 in a MGE-dependent step then allows for the recycling of mthsp70 and the continuation of the import process (Bolliger *et al.*, 1994; Laloraya *et al.*, 1994). Repeated cycles of mthsp70/Tim44 action then drive the completion of the import process.

Many aspects of the molecular ratchet model and motor models are identical (Fig. 1). The major difference in the models concerns how mthsp70 utilizes ATP to drive the unfolding of domains on preproteins that engage the import apparatus in a folded conformation. This is a critical question because the mechanism for the unfolding of proteins is likely to reflect how mthsp70 drives the overall protein translocation reaction. To resolve this issue biophysical experiments which measure the extent to which mthsp70 changes conformation after it hydrolyzes ATP and the force this movement generates need to be carried out. The rates at which domains on precursor proteins spontaneously unfold also need to be compared with rates of their import to determine if the kinetics for spontaneous unfolding at the import site are fast enough to account for import via the molecular ratchet mechanism.

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REFERENCES

Berthold, J., Bauer, M. F., Schneider, H.-C., Klaus, C., Dietmeier, K., Neupert, W., and Brunner, M. (1995). "The MIM complex mediates preprotein translocation across the mitochondrial inner membrane and couples it to the mt-hsp70/ATP driving system," *Cell* **81**, 1085–1093.

Blobel, G. (1980). "Intracellular protein topogenesis," *Proc. Natl. Acad. Sci. USA* **77**, 1496–1500.

Bolliger, L., Deloche, O., Glick, B. S., Georgopoulos, C., Jeno, P., and Kronidou, N., and Schatz, G. (1994). "A mitochondrial homolog of bacterial GrpE interacts with mitochondrial hsp70 and is essential for viability," *EMBO J.* **13**, 1998–2006.

Cyr, D. M., Stuart, R. A., and Neupert, W. (1993). "A matrix ATP requirement for presequence translocation across the inner membrane of mitochondria," *J. Biol. Chem.* **268**, 23751–23754.

Cyr, D. M., Lanser, T., and Douglas, M. G. (1994). "DNAJ-like proteins: molecular chaperones and specific regulators of hsp70," *Trends in Biochem. Sci.* **19**, 176–181.

Chen, W. J., and Douglas, M. G. (1987). "Phosphodiester bond cleavage outside mitochondria is required for the completion of protein import into the mitochondrial matrix," *Cell* **49**, 651–658.

Eilers, M., and Schatz, G. (1986). "Binding of a specific ligand inhibits import of a purified precursor protein into mitocondria," *Nature (London)* **322**, 228–232.

Gething, M.-J., and Sambrook, J. (1992). "Protein folding in the cell," *Nature (London)* **355**, 33–45.

Glick, B. S. (1995). "Can hsp70 proteins act as force generating motors? *Cell* **80**, 11–14.

Glick, B. S., Wachter, C., Reid, G. A., and Schatz, G. (1993). "Import of cytochrome b_2 to the mitochondrial imtermembrane space—the tightly folded heme-binding domain makes import dependent upon matrix ATP," *Protein Sci.* **2**, 1901–1917.

Hwang, S. T., and Schatz, G. (1989). "Translocation of proteins across the mitochondrial inner membrane, but not into the outer membrane, requires nucleoside triphosphates in the matrix," *Proc. Natl. Acad. Sci. USA* **86**, 8432–8436.

Kang, P.-J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A., and Pfanner, N. (1990). "Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins," *Nature (London)* **348**, 137–143.

Kronidou, N. G., Oppliger, W., Bolliger, L. Hannavy, K., Glick, B. S., and Schatz, G. (1994). "Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane," *Proc. Natl. Acad. Sci. USA* **91**, 12818–12822.

Laloraya, S., Gambill, B. D., and Craig, E. A., (1994). "A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation," *Proc. Natl. Acad. Sci. USA* **91**, 6481–6485.

Maarse, A. C., Blom, J. Grivell, L. A., Meijer, M. (1992). "MPI1, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria," *EMBO J.* **11**, 3619–3628.

Mayer, A., Neupert, W., and Lill., R. (1995). "Mitochondrial import: reversible binding of the presequence at the trans-side of the outer membrane drives partial translocation and unfolding," *Cell* **80**, 127–137.

Neupert, W., Hartl, F.-U., Craig, E. A., and Pfanner, N. (1990). "How do polypeptides cross mitochondrial membranes?" *Cell* **63**, 447–450.

Pfanner, N., and Meijer, M. (1995). "Protein sorting: pulling in the proteins," *Curr. Biol.* **5**, 132–135.

Rassow, J., Maarse, A. C., Krainer, F., Kubrich, M., Muller., Meijer, M., Craig, E. A., and Pfanner, N. (1994). "Mitochondrial protein import: biochemical and genetic evidence for the interaction of matrix hsp70 and the inner membrane protein Tim44," *J. Cell Biol.* **127**, 1547–1556.

Rassow, J., Hartl, F-U., Guiard, B., Pfanner, N., and Neupert, W. (1990). "Polypeptides traverse the membranes of mitochondria in an extended state," *FEBS Lett.* **275**, 190–194.

Schatz, G., and Dobberstein, B. (1996). "Common principles of protein translocation across membranes," *Science* **271**, 1519–1525.

Scherer, P. E., Krieg, U. C., Hwang, S. T., Vestweber, D., and Schatz, G. (1990). "A precursor protein partly translocated into yeast mitochondria is bound to a 70-kD mitochondrial stress protein," *EMBO J.* **9**, 4315–4322.

Schleyer, M., and Neupert, W. (1985). "Transport of proteins into mitochondria: translocational intermediates spanning contact sites between outer and inner membranes," *Cell* **43**, 339–350.

Schneider, H. C., Berthold, J., Bauer, M. F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994). "Mitochondrial Hsp70/Tim44 complex facilitates protein import," *Nature* **371**, 768–774.

Simon, S. M., Peskin, C. S., and Oster, G. F. (1992). "What drives the translocation of proteins?" *Proc. Natl. Acad. Sci. USA* **89**, 3770–3774.

Stuart, R. A., Cyr, D. M., Craig, E. A., and Neupert, W. (1994a). "Mitochondrial molecular chaperones: their role in protein translocation," *Trends Biochem. Sci.* **19**, 87–92.

Stuart, R. A., Gruhler, A., van der Klei, I. J., Guiard, B., Koll, H., and Neupert, W. (1994b). "The requirement of matrix ATP for the import of precursor proteins into the mitochondrial matrix and intermembrane space," *Eur. J. Biochem.* **220**, 9–18.

Ungermann, C., Neupert, W., and Cyr, D. M. (1994). "The role of hsp70 in conferring unidirectionality on protein translocation into mitochondria," *Science* **266**, 1250–1253.

Ungermann, C., Guiard, B., Neupert, W., and Cyr, D. M. (1996). "The DY and hsp70/MIM44 dependent reaction cycle driving early steps of protein translocation into mitochondria," *EMBO J.* **15**, 735–744.

Voos, W., Gambill, D. B., Guiard, B., Pfanner, N., and Craig, E. A. (1993). "Presequence and mature portion of preproteins strongly influence the dependence of mitochondrial protein import on the heat shock 70 protein in the matrix," *J. Cell Biol.* **123**, 109–118.

Wickner, W. (1994). "How ATP drives proteins across membranes," *Science* **266**, 1197–1198.

Xia, Z., and Matthews, F. S. (1990). "Molecular structure of flavocytochrome B2 at 2.4 Å resolution," *J. Mol. Biol.* **212**, 837–863.