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Editors

Paul A. Srere
Veterans Administration Medical Center-Dallas
Department of Biochemistry
University of Texas Southwestern Medical Center at Dallas
Dallas, Texas

Mary Ellen Jones
Department of Biochemistry
University of North Carolina
Chapel Hill, North Carolina

Christopher K. Mathews
Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon

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MEMBRANE TRANSLOCATION AND REFOLDING OF MITOCHONDRIAL PROTEINS

Franz-Ulrich Hartl and Walter Neupert

Institute of Physiological Chemistry, University of Munich,
Goethestrasse 33, 8000 München 2, FRG

ABSTRACT The biogenesis of mitochondria involves the transport of several hundred different proteins synthesized as precursors in the cytosol into the preexistent organelles. Membrane translocation occurs at translocation contact sites between outer and inner membranes through a hydrophilic membrane environment. These translocation sites can be envisoned as proteinaceous pores or channels and are present in limited number per mitochondrion. Precursor proteins arrested during translocation as membrane-spanning intermediates serve as molecular tools to study translocation contact sites. Recent evidence demonstrates that the membrane spanning portions of such intermediates can be in a rather extended conformation. This then implies that proteins have to refold once they reach the trans-side of the membranes to become functionally active. Refolding of imported proteins inside mitochondria and assembly into supramolecular complexes is not a spontaneous process but is mediated by a matrix-localized "assembly-complex" in an ATP-dependent manner. A major constituent of the mitochondrial machinery for the folding of proteins is the evolutionarily conserved heat-shock protein hsp60.

INTRODUCTION

Mitochondria, like chloroplasts, are assumed to have originated from procaryotic endosymbiotic ancestors. During evolution the organelles have lost their autonomy. Most mitochondrial proteins are coded for by nuclear genes and are

post-translationally imported into the preexistent organelles (1,2).

Over recent years transport of proteins into mitochondria has been dissected into a number of distinct steps which can be described as follows: i) Mitochondrial proteins are synthesized as precursors on cytosolic ribosomes. The interaction with 70 kD heat-shock proteins and probably with additional factors is required to keep precursors in a loosely-folded translocation-competent conformation. The requirement for nucleoside triphosphates of the import reaction is probably connected to these steps (3-10). ii) Precursors contain specific targeting signals (in most cases located at the amino-terminus as cleavable presequences) and interact via these signals with receptors at the surface of the outer mitochondrial membrane. Mitochondrial targeting sequences are positively-charged and are rich in hydroxylated amino-acids. Several of them have the potential to form amphiphilic helices upon insertion into membranes or when reaching contact with the phospholipids of membranes (11-13). iii) A component of the outer membrane, the "general insertion protein" (GIP), facilitates membrane insertion of precursor proteins (13). iv) Proteins of the outer membrane then directly reach their final location while proteins of all other submitochondrial compartments are directed into translocation contact sites between outer and inner membranes. This latter reaction is dependent on the electrical potential across the inner mitochondrial membrane (14-17). v) Amino-terminal presequences of precursors are cleaved during or after translocation by the matrix-localized metal-dependent processing peptidase (MPP) in cooperation with the processing enhancing protein (PEP) (18-20). vi) Following membrane translocation, precursors have to refold and in many cases to assemble into supramolecular complexes. Protein folding in the matrix is an ATP-dependent reaction mediated at the surface of the 14mer complex of the heat-shock protein hsp60 (21,22). vii) Proteins of the intermembrane space follow an evolutionarily conserved route to their target compartment. Their precursors are first translocated into the matrix and are then re-translocated across the inner membrane in a process which has similarity to bacterial protein export ("conservative sorting") (23-25). The mitochondrial inner membrane is unique in that it contains two independent machineries translocating proteins in opposite directions. Figure1 shows a model of the complex import and sorting pathway of cytochrome b_2 to the intermembrane space of yeast mitochondria.

In the present article we review recent advances made in our laboratory towards an understanding of the mechanisms of translocation of proteins across the mitochondrial membranes and their subsequent folding and assembly inside the organelles.

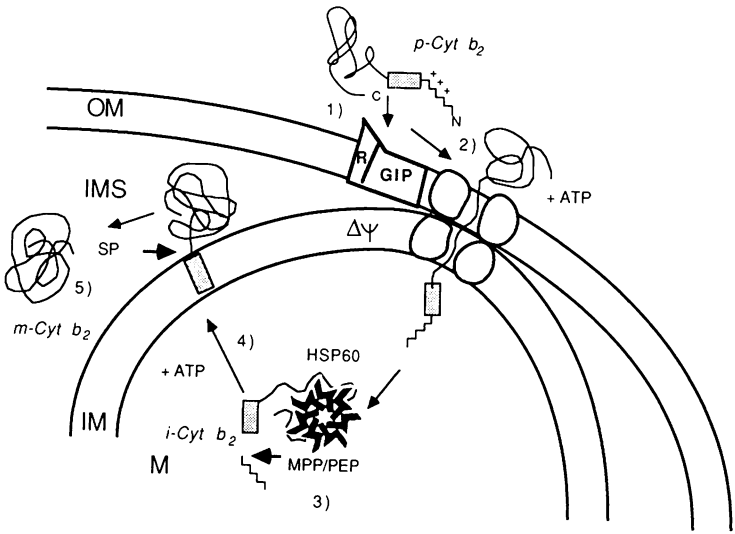

Figure 1: Working hypothesis for the translocation of cytochrome b_2 to the intermembrane space. (**1**). Cytochrome b_2 is made as a precursor (p-Cyt b_2) in the cytosol carrying an 80 amino acid residue presequence of bipartite structure. p-Cyt b_2 binds to a specific receptor (R) on the outer surface of the outer membrane, and, (**2**), is subsequently transported into translocation contact sites, via interaction with the "general insertion protein" (GIP) in the outer membrane. Transport into contact sites is dependent on the electrical component $\Delta\Psi$ of the total protonmotive force. Completion of translocation is independent of $\Delta\Psi$ but requires ATP probably for keeping precursor portions still outside the mitochondrion in an "unfolded" conformation. (**3**). p-Cyt b_2 interacts with the hsp60-complex in the matrix and cleavage of the first part of the presequence (the positively-charged mitochondrial targeting sequence) occurs by the action of MPP in cooperation with PEP resulting in the formation of intermediate-sized cytochrome b_2 (i-Cyt b_2). (**4**). The prepeptide of i-Cyt b_2 (which resembles a bacterial type

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MITOCHONDRIAL TRANSLOCATION CONTACT SITES

Most imported mitochondrial proteins have to be translocated at least partially into or across the inner membrane Transport across outer and inner membranes occurs in a single step at translocation contact sites. Morphological studies have revealed these sites of close contact between the mitochondrial membranes for many years (26). First biochemical evidence tha translocation of proteins proceeds via contact sites came from *i vitro* experiments in which precursor proteins spanning both mitochondrial membranes were accumulated as translocation intermediates (14). Precursor proteins which had antibodies bound to carboxy-terminal parts of the protein were arrested during translocation in a position reaching into the matrix with the amino-terminus (which was proteolytically processed) but with other parts of the molecule still being outside the mitochondrion where they were accessible to added protease. Apparently, the two membranes had to be close enough to be spanned by a single polypeptide chain. Performing the import reaction at low temperature or at decreased levels of nucleoside triphosphates (NTPs) also caused arrest of precursors in translocation contact sites (8,9,14). We assume that these procedures render the mature protein part of the precursor incompetent for translocatio by conferring a more stably-folded structure. NTPs are probably required to keep precursors in the cytosol in a loosely-folded, translocation competent conformation via the action of 70 kD heat-shock proteins and maybe additional factors (3-10). As demonstrated by immuno-gold labeling of translocation intermediates the biochemically-defined translocation contact sites are identical with the morphologically-described sites of close contact between the two membranes (15).

Contact sites appear to be stable structures. Submitochondrial fractions enriched in contact sites could be

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export signal) directs the re-translocation of the protein back across the inner membrane. ATP is probably necessary for the release of i-Cyt b_2 from hsp60. (**5**). Cleavage by a second processing enzyme (SP) at the outer surface of the inner membrane generates the mature-sized protein (m-Cyt b_2), a soluble component of the intermembrane space. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; M, matrix.

obtained by sonication of isolated mitochondria and sucrose gradient centrifugation. Low concentrations of digitonin, a sterol specific detergent, were able to remove large parts of the outer membrane except at regions where outer and inner membranes were attached to each other (15). Precursors spanning the mitochondrial membranes as translocation intermediates were extractable with protein denaturants such as urea or alkaline pH indicating that the membrane spanning intermediates were in a hydrophilic, probably proteinaceous environment (27).

Only the transport of precursors from the cytosol into translocation contact sites is dependent on the membrane potential across the inner membrane. The completion of translocation across the inner membrane is independent of the membrane potential but requires NTPs for keeping precursor portions still outside the mitochondrion in an "unfolded" conformation (see above) (8,14). The insertion of the positively-charged presequences into the inner membrane seems to be the membrane-potential dependent step of the import pathway. The electrical component $\Delta \Psi$ of the total protonmotive force and not the chemical component Δ pH is required (17). The role of $\Delta \psi$ is unclear. It might be speculated that the membrane potential (negative inside) exerts an electrophoretic effect on the positively-charged regions of the precursor proteins.

Fusion Proteins to Study Translocation Contact Sites.

In collaboration with B. Guiard (Gif-sur-Yvette) we have recently designed a mitochondrial fusion protein which can be used as a molecular tool to study translocation contact sites. The amino-terminal 167 amino acid residues of the precursor of yeast cytochrome b_2 were fused to the amino-terminus of the complete sequence of mouse dihydrofolate reductase ($pb_2$167-DHFR) (16). In this construct the cytochrome b_2 part and the DHFR moiety fold independently of each other. Binding of the folate antagonist methotrexate stabilizes the folded structure of DHFR (28). Under these conditions pb_2-DHFR is only partially translocated and is arrested as translocation intermediate spanning the two membranes. The cytochrome b_2 part of the fusion protein reaches into the matrix where cleavage by the processing peptidase occurs while the folded DHFR stays outside of the outer membrane and can be removed by externally added protease. In the presence of methotrexate DHFR is not digested by protease

and is recovered in the soluble supernatant of the reaction. If the methotrexate is removed from the translocation intermediate by washing of the reisolated mitochondria the DHFR moiety of the construct is unfolded at the surface of the outer membrane probably by the action of a proteinaceous component and the fusion protein is completely translocated into the interior of mitochondria.

The fusion protein pb_2-DHFR was used in the presence of methotrexate to titrate translocation contact sites of mitochondria (16). Around 70 pmoles of translocation intermediates had to be accumulated per mg of mitochondrial protein to reach saturation. It was calculated that a single mitochondrion accomodated about 4000 translocation intermediates. Morphometric analysis of the mitochondria of *Neurospora crassa* revealed that the total area of close contact between the mitochondrial membranes corresponded to about 7% of the total outer membrane surface. At present it is unknown whether contact sites are point-shaped structures or whether they are narrow stripes extending over longer distances at the origin of cristae. Based on the three-dimensional structure of DHFR we estimated that saturating amounts of translocation intermediates occupied about 1% of the outer membrane surface thus potentially leaving enough room in contact sites for proteinaceous components involved in the translocation process. Mitochondria which had accumulated saturating amounts of the translocation intermediate were unable to import the precursor of the ß subunit of F_1ATPase and the precursor of the Rieske Fe/S protein of complex III which is localized at the outer surface of the inner membrane. The rates of import of these proteins decreased in correlation to the degree of presaturation of mitochondria with contact site intermediates. Apparently, translocation contact sites occur in limited number. The same translocation sites are used by different precursor proteins destined to different submitochondrial compartments (16,29).

How many amino acid residues are contained in the segment of a precursor which spans the two membranes at translocation contact sites? To address this question a series of fusion proteins derived from the $pb_2$167-DHFR construct were engineered by stepwise shortening its cytochrome b_2 part (Rassow et al., in preparation). In the absence of methotrexate also the shortest fusion protein, $pb_2$47-DHFR, was readily translocated across the mitochondrial membranes and

proteolytically processed. In the presence of methotrexate, however, $pb_2$76-DHFR was the shortest construct which was still able to span the two membranes and to expose the proteolytic cleavage site at position 30 of the presequence to the matrix-localized processing peptidase. Shorter fusion proteins did no more accomplish stable accumulation in translocation contact sites. This allowed the conclusion that about 46 amino acid residues were sufficient to span the two mitochondrial membranes at contact sites. On electron micrographs of isolated mitochondria the distance from outer surface of outer membrane to inner surface of inner membrane at membrane contacts was determined to be 15-17 nm. This could correspond well to the diameter of two protein rich membranes. Although the two lipid bilayers always appeared to be separated by a narrow gap we cannot completely rule out the possibility that fusions between the bilayers might occur at very distinct areas. Nevertheless, our data would indicate that the region of a stable translocation intermediate spanning contact sites is essentially devoid of tertiary structure. The conformation of the spanning polypeptide might be even more extended than α-helical. The necessity for cytosolic precursor proteins to assume an "unfolded" conformation may therefore directly reflect mechanistic requirements at the molecular level of the translocation process itself.

ROLE OF HSP60 IN REFOLDING AND ASSEMBLY OF MITOCHONDRIAL PROTEINS

Once translocated across the mitochondrial membranes proteins remaining in the mitochondrial matrix have to refold and in many cases to assemble into supramolecular complexes (22). Several intermembrane space proteins are first imported into the matrix and are then re-exported across the inner membrane (23-25). Their precursors carry bipartite amino-terminal targeting sequences whose second parts have characteristics of bacterial export signals (24,30). It has to be assumed that these proteins have to remain in a loosely-folded conformation prior to the second membrane translocation event. Very likely this is also the case for inner membrane proteins including those coded for by the mitochondrial genome, which insert into the membrane from the matrix space.

In contrast to the view generally held so far, folding and assembly of imported mitochondrial proteins does not occur spontaneously, but is mediated by proteinaceous components. The recently described matrix-localized stress-protein hsp60 plays an essential role in these processes (22). Hsp60 is a constitutively expressed heat-shock protein which is functionally and structurally related to the *E. coli* heat-shock protein groEL and to the α-component of the Rubisco subunit-binding protein of chloroplasts (21,22,31). Hsp60, the Rubisco binding protein and groEL have been grouped into a subclass of "molecular chaperones" termed "chaperonins" (31), components assisting in oligomeric protein assembly. Interestingly, the "chaperonins" reside in macromolecular 14mer complexes consisting of two 7-mer rings one put on top of the other.

In an attempt to identify components of the mitochondrial machinery for protein translocation and assembly, Horwich and colleagues selected temperature-sensitive yeast mutants with specific defects in mitochondrial protein import (19,22). One nuclear mutation, mif4 (mif for mitochondrial import function), affecting the gene for hsp60 enabled the identification of hsp60 function (22). At the non-permissive temperature mutant cells showed a deficiency in the assembly of several mitochondrial proteins of matrix, inner membrane and intermembrane space. For example, the precursor of the ß- subunit of the F_1-ATPase was completely translocated across the mitochondrial membranes and proteolytically processed but failed to assemble into the FoF_1-ATPase complex. Likewise, the matrix enzyme ornithine transcarbamylase was not able to form the functionally active homotrimer. Proteins of the intermembrane space, such as cytochrome b_2 and the Rieske Fe/S protein, apparently misfolded in the matrix space and did not reach their target compartment. It was found that in the mutant the structural integrity of the hsp60 complex was affected. Hsp60 complex isolated from cells grown at the non-permissive temperature appeared to have denatured and sedimented into a low speed pellet.

Imported Proteins Fold in an ATP-dependent Reaction.

We have now analyzed the sequence of steps during the refolding of proteins imported into the mitochondrial matrix. Using a fusion protein consisting of the mitochondrial presequence of

subunit 9 of Fo-ATPase and the complete mouse DHFR we were able to determine the folding state of the imported protein by measuring the protease resistance of the DHFR moiety (Ostermann et al., in preparation). Unfolding of the DHFR part occurring at the surface of the outer membrane has been shown to be the rate limiting step for the translocation of similar fusion proteins across the mitochondrial membranes. To achieve fast, ATP-independent membrane translocation we therefore used precursor preparations in our import experiments which were artificially unfolded by incubation in 8M urea. Under these conditions, the kinetics of refolding of the imported protein in the matrix were slower than its translocation via contact sites. If mitochondria were depleted of ATP prior to import, the refolding of DHFR was completely blocked ("folding arrest"). The incompletely folded fusion protein could be extracted from the matrix of mitochondria as a high molecular weight "assembly complex" which migrated on sizing columns with an apparent molecular weight of 800 kD. Using non-denaturing polyacrylamide gel electrophoresis hsp60 was identified as a major constituent of this "assembly complex". In the absence of ATP the fusion protein associated to hsp60 was completely sensitive towards digestion by protease while the hsp60 complex itself was protease resistant. Apparently, the incompletely folded polypeptide chains of the imported protein were exposed at the surface of hsp60. Readdition of ATP to the assembly complex initiated folding of the associated polypeptide and release from hsp60. GTP and the non-hydrolyzable ATP analog AMP-PNP were ineffective. A very similar sequence of reactions was observed for authentic imported mitochondrial proteins including the ß-subunit of F_1-ATPase and the Rieske Fe/S protein.

So far it is unknown how hsp60 functions in folding and assembly of mitochondrial proteins. It seems obvious that hsp60 recognizes some structural motif of the "unfolded" polypeptide chains entering the mitochondrial matrix via contact sites. One important function of the "chaperonin" hsp60 could be to capture these polypeptides thereby preventing their aggregation in the high protein concentration of the matrix space. Both, the hsp60 homologue groEL and the 70 kD heat-shock proteins are weak ATPases. The initial binding of the protein substrate to hsp60 is ATP-independent. As proposed for the interaction of proteins with 70 kD heat-shock proteins (32), ATP-hydrolysis by hsp60 could cause a conformational change of the hsp60 complex

which is transferred to the associated polypeptide chain(s). This could loosen the interaction with the unfolded polypeptide thus allowing for its ordered, domain-wise folding. At the same time, the protein substrates interacting with hsp60 could expose complimentary surfaces facilitating the assembly with other subunits to homo- or heterooligomeric complexes. It is unclear whether additional components are involved in these processes. In *E. coli* the groES protein has been shown to cooperate with groEL in functions such as assembly of prokaryotic ribulose-bisphosphate carboxylase and assembly of phage particles (33,34). An equivalent to groES has not been detected in either mitochondria or chloroplasts.

PERSPECTIVES

Despite the progress made over the recent years, the mechanisms involved in translocation of proteins across biological membranes are still enigmatic. The machinery for protein translocation has not yet been identified in any of the well studied membrane systems. Clearly, one of the major tasks for the future will be the isolation of the mitochondrial translocation contact sites and their molecular characterization. To achieve this goal, the mitochondrial fusion proteins which can be accumulated as stable membrane-spanning intermediates are currently being used as "molecular handle".

It is an emerging theme in present-day cell biology that the folding and assembly of proteins, for a long time been viewed as spontaneous processes, are protein catalyzed reactions in every cellular compartment *in vivo* . For example, the immunoglobulin heavy chain binding protein (BiP) of the endoplasmic reticulum appears to have functions very similar to those described for the mitochondrial hsp60 (32). These findings may also be of considerable biotechnological relevance. One might speculate that in the future components like hsp60 or the groE proteins will be used as "folding catalysts" to solve the problem of active reconstitution of proteins obtained by overexpression in bacteria. However, a more detailed understanding of how these components function at the molecular level will be required to make this a feasible approach.

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