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Membrane Biogenesis and Protein Targeting

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General and exceptional pathways of protein import into sub-mitochondrial compartments

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1. *The general pathways for protein import into sub-mitochondrial compartments*

In the past few years, considerable progress has been made to elucidate the mechanisms of how nuclear-encoded proteins are imported into and sorted within mitochondria (for detailed reviews, see [1–5]). The consensus pathways for protein sorting into the different mitochondrial compartments are depicted in Fig. 1. Nuclear encoded mitochondrial proteins are synthesized on cytoplasmic ribosomes as precursors which usually contain N-terminal targeting (signal) sequences. These signal peptides are positively charged and have the potential to form amphipathic α -helices [6]. Some precursor proteins, however, lack such cleavable pre-sequences, such as those destined for the mitochondrial outer membrane (OM). To avoid premature folding or aggregation in the cytosol, the precursors are transiently bound to cytosolic chaperone proteins which have been shown to belong to the heat shock protein 70 (hsp70) class [7]. Dissociation from hsp70 requires the hydrolysis of ATP which, in many cases, is considered to be a rate-limiting step for translocation.

Specific interaction of most precursor proteins with the OM is mediated by protease-sensitive surface receptors. *Neurospora crassa* MOM19 acts as the receptor for the majority of proteins analyzed so far [8], whereas the participation of *N. crassa* MOM72 has been demonstrated only in connection with the import of the ADP/ATP carrier [9]. Its yeast counterpart, MAS70, appears to have a broader substrate specificity [10,11]. Translocation through or insertion into the OM is catalyzed by a number of proteins which are associated in a complex in the OM (Fig. 1). This complex (termed the receptor/GIP complex) has been identified by co-immunoprecipitation with the surface receptors MOM19 or MOM72 which are part of the

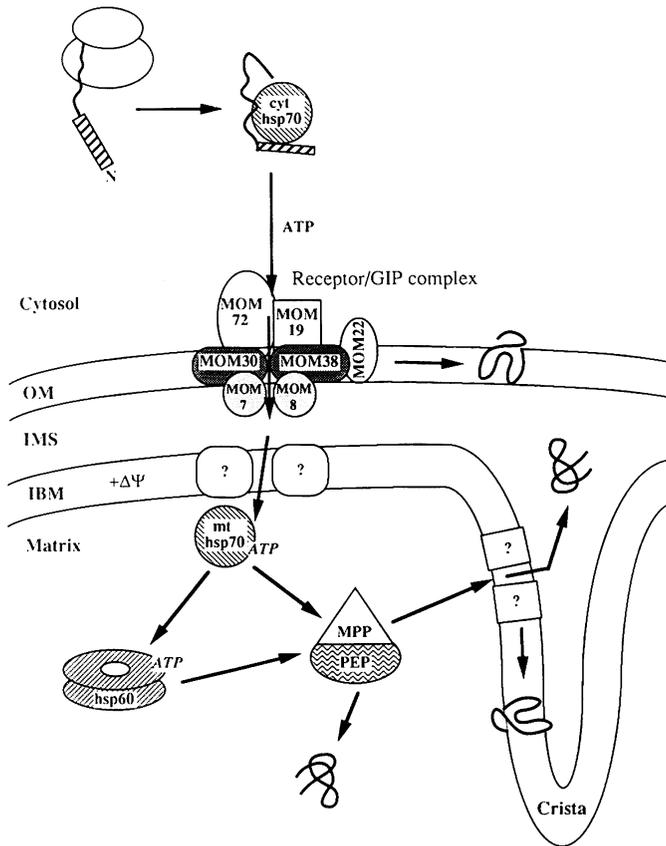


Fig. 1. The general pathways of protein import into sub-mitochondrial compartments. See text for a detailed explanation. It should be noted here that we do not know the specific interactions between the individual components of the receptor/GIP complex in the OM. However, according to their protease-resistance, MOM38, MOM30, MOM8, and MOM7 are deeply inserted into the OM or are even exposed to the IMS, while MOM19 and MOM72, as surface receptors, and MOM22 face the cytosol. Consistent with this observation it has been demonstrated that the former set of proteins forms (at least part of) GIP, a site where precursor proteins like AAC are already deeply inserted into the OM and are protected from externally added protease. Aside from its co-immunoprecipitation with MOM19, the role of MOM22 is unknown at present. For the sake of clarity, the second proteolytic step occurring in the IMS after export of proteins from the matrix has been omitted. Proteins inserted into the OM normally do not contain cleavable signal sequences (see text). IBM, inner boundary membrane; MOM x , mitochondrial outer membrane protein with a molecular mass of x kDa. MPP, matrix processing peptidase; PEP, processing enhancing protein; Cyt and mt hsp70, cytosolic and mitochondrial isoforms of heat shock protein of 70 kDa; Hsp60, heat shock protein of 60 kDa; ?, unknown components of the protein import and export apparatus of the IM.

complex [12]. One of its components, *N. crassa* MOM38, is part of a translocation site in which the precursor proteins are already deeply inserted in the OM and thus are

resistant to externally added protease. Since this site is used by most precursor proteins, it has been termed general insertion pore (GIP) [13]. The yeast counterpart of MOM38, ISP42, is essential for cell growth [14]. Both functional and structural analysis of the receptor/GIP complex has been achieved by crosslinking the precursor of the ADP/ATP carrier (AAC) to various components of the complex [15]. Crosslinking of AAC arrested at different stages of its passage through the OM [16] allowed the components acting early and late in the OM translocation reaction to be defined. Receptor-bound AAC could be crosslinked to MOM19 and MOM72, whereas AAC arrested at the level of GIP could be shown to react efficiently with MOM7, MOM8, and with MOM30 proteins thus identifying new members of the complex.

Proteins destined for the intermembrane space (IMS), the inner membrane (IM), and the matrix become further translocated through the IM at sites of close contact between OM and IM [17]. Under *in vivo* conditions, more than 90% of the OM surface is in close contact with parts of the IM, the inner boundary membrane, which is competent for protein import (Fig. 1). Only a fraction of this area (10%) is made up of the morphological contact sites which comprise stable attachments of OM and IM even after shrinking of the IM. The initial interaction of the precursor proteins with the IM is mediated through an N-terminal signal sequence, and translocation is dependent on a membrane potential across the IM. Nothing is known so far about the nature of the components of the IM import machinery. The action of mitochondrial hsp70 (located in the matrix) and ATP hydrolysis are thought to be needed to pull the proteins into the matrix and are required for subsequent folding of matrix proteins [18]. N-terminal signal sequences are cleaved off by the matrix processing peptidase consisting of two components (MPP and PEP in *N. crassa*, MAS1 and MAS2 in yeast). Some of the incoming proteins also interact with hsp60 in the matrix which assists in folding and assembly of the incoming proteins [19–21].

Proteins of the IM or the IMS are then retargeted into the IM. A second signal sequence which shows a close similarity to bacterial export signals mediates this interaction. IM proteins become inserted into the IM, while proteins destined for the IMS are completely translocated through the IM. Finally, the second signal sequence becomes cleaved, e.g., by the inner membrane protease [22], whereafter IM proteins are functionally assembled, and IMS proteins are released as soluble proteins into the IMS. Since this membrane transit step mechanistically resembles the path taken by proteins in the bacterial ancestors of mitochondria, it has been termed the 'conservative sorting pathway' [2]. Nothing is known so far about the re-translocation (export) apparatus in the IM. One may speculate though that the components of such a translocation complex may bear some similarity to the members of the protein translocase identified in the plasma membrane of bacteria [23].

2. Exceptional pathways of protein import

A few exceptions to the general protein import pathways into the sub-mitochondrial compartments have been described so far. In these cases, only some steps or components of the general pathways are used while others are bypassed or alternative routes are taken. For example, import of yeast cytochrome *c* oxidase subunit Va, a protein of the IM (Cox Va) [24], is independent of protease-sensitive surface receptors in the OM. The protein enters the OM obviously at a later stage, most likely at the GIP stage, thereby circumventing the participation of surface receptors. The import of Cox Va is independent of surface receptors, and is similar to the situation found for MOM19 (see below) and for a low-efficiency bypass import of several proteins that was observed even after the surface receptors had been completely degraded [25]. For delivery of the Cox Va precursor protein to the OM, hsp70 seems not to be involved, since comparatively low levels of ATP are needed, and import occurs efficiently even at low temperatures. On the other hand, import of Cox Va is reported to share the features of the general pathway in that (i) translocation occurs via translocation contact sites, (ii) it needs a membrane potential for translocation, and (iii) its pre-sequence is cleaved after import.

A non-conservative import pathway into the IM without a transient passage through the matrix has been reported for the *N. crassa* ADP/ATP carrier protein (AAC)[26]. As mentioned above, AAC is so far the only known precursor that uses MOM72 instead of MOM19, as an import receptor. From MOM72, AAC is transferred into the protease-resistant location of GIP. Dependent on a membrane-potential across the IM, but independent of ATP and hsp60, as found for proteins of the general pathway, AAC is directly inserted into the IM and functionally assembled [26]. In vivo experiments with subunit VI of yeast QH₂ cytochrome *c* reductase (SubVI) suggest a direct transfer of this protein to the outer face of the IM [27]. The import into mitochondria is thought to be governed by its unusual N-terminal pre-sequence which is 25 amino acids long, but, in contrast to normal signal sequences, is highly negatively charged. Fusion proteins of the pre-sequence with the mature part of superoxide dismutase (SOD) as a reporter protein (pre-SubVI-SOD) were transported into the IMS as analyzed by sub-mitochondrial fractionation after in vivo import into mitochondria [27]. Furthermore, pre-SubVI-SOD failed to complement a mutant in the matrix-localized SOD also suggesting no transport of the fusion protein to the matrix. Thus, a negatively charged pre-sequence is also able to direct proteins into mitochondria, and seems to contain the information that precludes transfer of the attached protein across the IM.

Import of AAC and a few other mitochondrial proteins is unusual with respect to the absence of a cleavable signal sequence at the N-terminus of these proteins. In some cases, the sorting signals have been located somewhere in the mature region of the proteins, usually close to the N-terminus. Examples with non-cleavable signal sequences include the OM proteins MAS70 of yeast, *N. crassa* MOM72, and porin

(see above), as well as AAC, 3-oxoacyl-CoA thiolase (reviewed in [1]), and some ribosomal proteins [28]. Many of these ribosomal proteins might lack a separate signal, since this class of proteins is usually highly positively charged, and thus already contains sequences that are similar to standard signal sequences.

In contrast to these proteins which use at least parts of the consensus pathway, cytochrome *c*, a soluble protein of the IMS, has developed quite an exceptional pathway that has no connection to the general routes. Cytochrome *c* is imported through the OM without the need for protease-sensitive surface receptors or the OM import receptor complex described above (for a review see [29]). Rather, cytochrome *c* utilizes a high-affinity interaction with cytochrome *c* heme lyase (CCHL) for its specific import into mitochondria. CCHL covalently attaches the heme group to cytochrome *c* and is located in the IMS at the outer face of the IM. Heme attachment may already occur when cytochrome *c* is still bound to the OM in a trans-membrane fashion [30]. Folding of the protein chain around the hydrophobic heme group may then lead to the release of functional holocytochrome *c* into the IMS. Thus, CCHL, during the biogenesis of cytochrome *c*, provides a dual function: (i) as an import receptor specific for cytochrome *c*, and (ii) as an enzyme which covalently adds the prosthetic group to the incoming cytochrome *c* thus rendering the import reaction irreversible.

The study of exceptional import pathways into mitochondria is rewarding in many aspects. First, it provides information about new components involved in protein transport across membranes. Second, it helps to elucidate the function of certain components of the general pathway, e.g., the determinants of recognition by these components. Third, it may serve to understand how import into mitochondria is regulated in order to achieve a balance between imported material and import catalysts. We have studied the import pathways of two proteins, the OM protein MOM19 and CCHL of the IMS, in some detail. Both proteins are imported along interesting, novel pathways into mitochondria.

3. MOM19 is imported into the OM without the aid of surface receptors

MOM 19 is the major surface receptor for protein import into *N. crassa* mitochondria which is used by most precursor proteins analyzed so far [8]. It was interesting to see how MOM19 itself is delivered to mitochondria. To study its import pathway, we first cloned the cDNA and determined the protein sequence [31]. As expected for an OM protein, MOM19 does not contain a cleavable N-terminal signal sequence. Residues 42–59 may form a highly positively charged, amphipathic α -helix and may serve as an internal, uncleaved import signal. The first 26 amino acids are hydrophobic and may represent the membrane anchor for the protein. The rest of the protein is hydrophilic and most likely protrudes into the cytosol.

For the analysis of the *in vitro* import of MOM19 into isolated *N. crassa*

mitochondria, we made use of a property of endogenous MOM19. Treatment of mitochondria with elastase produces characteristic fragments of MOM19. When MOM19 precursor was imported in vitro from reticulocyte lysate, the same proteolytic pattern as that with endogenous MOM19 was produced after elastase treatment. MOM19 in reticulocyte lysate or MOM19 bound to the surface of mitochondria did not give rise to the specific proteolytic pattern, but was further degraded under the conditions used. These results indicate that imported MOM19 was functionally inserted into the OM [31].

Which components are involved in the insertion of MOM19 into the OM? When the mitochondria were pre-treated with protease to degrade the endogenous surface receptors, namely MOM19 and MOM72, the import of MOM19 was unaffected. This result shows that MOM19 does not utilize one of the protease-sensitive surface receptors in the OM for its own import. How, then, does MOM19 become specifically imported into mitochondria and does not associate with other cellular membranes like microsomes [31]? One possibility is that MOM19 enters the mitochondria via MOM38 with which it forms a tight complex [12]. Since *N. crassa* MOM38 is inaccessible to protease and its function cannot be blocked by antibodies, we made use of the yeast homolog of MOM38, namely ISP42, which in contrast is accessible to antibodies [32]. *N. crassa* MOM19 was imported into yeast mitochondria and functionally assembled into the receptor complex as shown by co-immunoprecipitation of MOM19 with anti-ISP42 antibodies. This heterologous import and assembly of MOM19 also suggests that the receptor complexes of yeast and *N. crassa* are functionally very similar. Mitochondria which were pre-blocked with anti-ISP42 antibody failed to import MOM19 [31], demonstrating that ISP42 is directly involved in the import of MOM19. Since the *N. crassa* homolog of ISP42, MOM38, is a component of GIP, these results suggest that MOM19 enters the mitochondria directly at the level of the GIP site thereby bypassing the surface receptors. The same conclusion can be drawn from import experiments with yeast mitochondria pre-treated with protease at concentrations which degraded the receptors but not ISP42. Import of MOM19 into such mitochondria remained unaffected, when import of other, receptor-dependent proteins had already vanished.

For other components of the mitochondrial protein import machinery it has been shown that they participate in their own biogenesis. Examples include mitochondrial hsp60 [20], hsp70 [33], and the matrix processing peptidase [34]. Why does MOM19 circumvent the use of protease-sensitive surface receptors like MOM19 itself? The receptor-independent pathway entering the OM at MOM38/GIP might represent an evolutionary remnant form of mitochondrial protein import allowing specific import without the need of surface receptors. The invention of surface receptors might then serve a dual function: (i) it increases the specificity for import by the introduction of another checkpoint in addition to MOM38/GIP; (ii) it increases the efficiency and the rate of import considerably. In line with these assumptions, a low efficiency import of several proteins into mitochondria was found even after the surface receptors had

been removed by protease digestion [25]. Most likely, these proteins enter the mitochondria at a later step.

4. *Cytochrome c heme lyase is imported directly through the OM via a non-conservative sorting pathway*

N. crassa cytochrome *c* heme lyase (CCHL) is localized in the IMS, where it is found associated with the outer face of the IM [30]. The enzyme catalyzes the covalent attachment of a reduced heme group to cytochrome *c*, and it serves as a high-affinity receptor for cytochrome *c* during its import across the OM [29]. *N. crassa* CCHL is about 60% homologous (40% identity on amino acid level) to both yeast CCHL and to yeast cytochrome *c*₁ heme lyase (CC₁HL [35,36; A. Haid, pers. commun.]). All three heme lyases lack cleavable N-terminal signal sequences. We have analyzed the import pathway of *N. crassa* CCHL in vitro and found an interesting import mechanism which, on the one hand, shares characteristics of the general pathway (see Fig. 1), and on the other hand, uses novel ways to reach the IMS.

CCHL synthesized in an in vitro transcription-translation system efficiently and rapidly associates with mitochondria [37]. During further incubation, CCHL becomes translocated into the organelles, as assayed by protection of imported CCHL against digestion by externally added protease. As revealed by digitonin fractionation of mitochondria, imported CCHL is correctly localized to the IMS, its functional environment. Here, it is bound to membranes, and thus behaves indistinguishably from the endogenous protein [30] and its enzyme activity [38].

CCHL does not require free ATP for import into mitochondria, an observation which is in sharp contrast to what has been reported for other mitochondrial precursor proteins. Except for a requirement of ATP in the matrix (see above), ATP hydrolysis is needed for the dissociation of the precursor proteins from cytosolic hsp70 proteins. Hsp70s bind to precursor proteins in order to keep them competent for translocation by stabilizing an unfolded conformation [7]. Dissociation from hsp70 is assumed to be the rate-limiting step for translocation. For the import of CCHL, however, several observations argue against a participation of cytosolic hsp70 proteins [37]. (i) CCHL is rapidly imported into the IMS. Even at 0°C import occurs with a half-time of 10 min, and thus is much faster than observed for other precursors. (ii) The time course and the efficiency of the import are unchanged when CCHL is denatured in 8 M urea and imported into mitochondria after rapid dilution of the denaturant. Usually, unfolding of the precursor proteins and disruption of the interaction with hsp70 accelerates the import kinetics markedly. (iii) CCHL import occurs independently of the presence of free ATP. When ATP is hydrolyzed by apyrase (an ATP- and ADP-hydrolyzing enzyme activity from potato), import of CCHL is unaffected, while the import of control proteins like the β -subunit of the F₁-ATPase is completely inhibited. CCHL imported in the absence of

ATP is still correctly localized in the IMS indicating that CCHL is not simply trapped in an intermediate stage during import. The observation that CCHL import does not involve both hsp70 and the hydrolysis of free ATP may be related to the unusually high content of proline residues (11.6%) which might keep the protein in an extended, import-competent structure without the aid of chaperone proteins.

For translocation through the OM, CCHL uses the receptor/GIP complex. Import of CCHL was completely abolished when mitochondria were pre-treated with protease indicating that protease-sensitive receptors mediate the entry of CCHL into the OM [37]. To find out which specific receptor is used, mitochondria were pre-incubated with immunoglobulin G (IgG) against MOM19, MOM72, and as a control, porin. Subsequent import of CCHL was specifically reduced by 80% in mitochondria that had been blocked with anti-MOM19 antibodies as compared to unblocked mitochondria, while the other antibodies did not reduce the import efficiency. These results demonstrate that CCHL, as most other mitochondrial precursor proteins, uses the main receptor of *N. crassa* mitochondria, MOM19, for initial interaction with mitochondria. Furthermore, competition experiments with chemical amounts of import-competent porin demonstrate that CCHL uses the same OM import machinery, namely the receptor/GIP complex which is effective for most other mitochondrial precursor proteins [37].

How does CCHL reach the IMS after transit through the OM? Two possibilities can be envisaged. Either, the protein is further translocated via contact sites through the IM and the matrix along the conservative sorting pathway (Fig. 1), or it enters the IMS directly after its passage through the OM (Fig. 2A). The first possibility seems unlikely from the fact that CCHL lacks the usual bipartite, cleavable pre-sequence (as found in, e.g., cytochrome c_1 - and cytochrome b_2 -precursors). The positively charged signal sequence is thought to mediate the interaction with the IM. For insertion and translocation through the IM, a membrane potential has been shown to be essential [3,39]. Consistent with the view that CCHL translocates directly into the IMS, its import into mitochondria occurs independently of the existence of a membrane potential [37]. Depletion of the membrane potential by uncouplers like CCCP did not influence the import of CCHL into mitochondria. The same result was obtained when either ionophores (valinomycin) or inhibitors of the electron transport chain and of the F_1F_0 -ATPase (antimycin A and oligomycin, respectively) were used to prevent the formation of a membrane potential. These results strongly suggest that CCHL does not pass through the IM during its import into mitochondria. Rather, it reaches the IMS directly after translocation through the OM import channel via a non-conservative sorting pathway.

The fact that CCHL requires neither ATP hydrolysis nor a membrane potential as energy sources for import, raises the important question of what energetically drives the import reaction. Three possibilities may be anticipated. (i) The driving force for import may come from folding of CCHL within the IMS. It will be interesting to see whether such folding reaction is catalyzed by yet to be discovered factors. (ii)

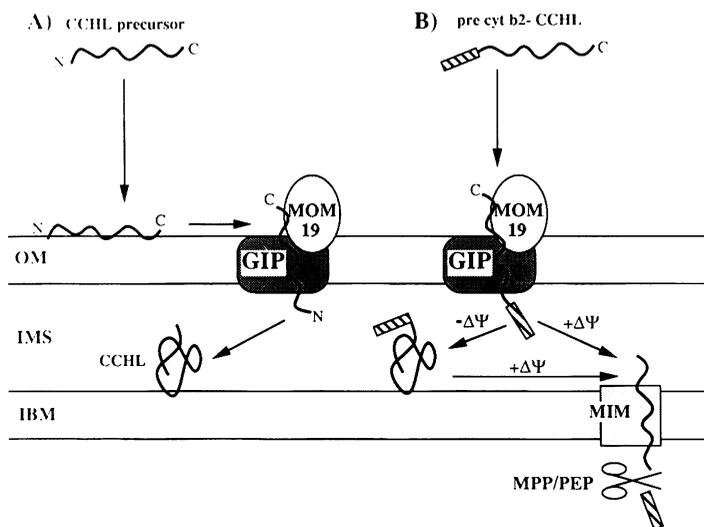


Fig. 2. (A) The non-conservative import pathway of CCHL across the OM and (B) the two-step import mechanism of CCHL carrying an N-terminal matrix targeting sequence. See text for a detailed explanation. Pre cyt b₂-CCHL represents CCHL with the positively charged pre-sequence of cytochrome *b*₂ (shown by the hatched rectangle) attached to its N-terminus; MIM, mitochondrial inner membrane import apparatus; GIP, general insertion pore. All other abbreviations are as in Fig. 1.

Alternatively, CCHL may be pulled into the IMS by specific interaction with a binding partner. Since CCHL avidly binds to liposomes, such an interaction may include binding to lipid molecules in the IM and OM. (iii) Finally, it cannot be excluded on the basis of the previous experiments (see above) that the hydrolysis of miniscule amounts of factor-bound ATP, inaccessible to digestion by apyrase, may drive the import. It should be mentioned here that MOM38 contains an ATP binding motif [12], and thus may be a candidate for such an ATP binding factor. These questions are now open to direct experimental approach. Recently, an *in vitro* import system with purified OM vesicles has been developed. CCHL as well as OM proteins are imported into these vesicles in a receptor-dependent fashion (A. Mayer, R. Lill and W. Neupert, unpublished results). The ability of OM vesicles to import CCHL nicely confirms the conclusions drawn above that the IM is not involved in the passage of CCHL into the IMS.

Which signals guide CCHL along its path to the IMS and why is further translocation through the IM precluded? The import signal in CCHL is unknown. Experiments with antibodies raised against the N- and C-terminus of CCHL show that the N-terminal half of CCHL inserts into the OM first and thus must contain the information for OM interaction (R. Lill and W. Neupert, unpublished results). Both antibodies efficiently block translocation of CCHL. When import is inhibited by the C-terminal antibodies, specific N-terminal fragments of CCHL are produced after

protease digestion. Occurrence of these fragments is dependent on functional MOM19 protein and is not observed with either control or N-terminal antibodies. These experiments show that CCHL with an attached C-terminal antibody may form a membrane-spanning translocation intermediate in the OM. From the size of the observed fragments of 34, 28, and 22 kDa, it can be concluded that the N-terminal half of the protein (i) enters the OM first, and (ii) contains the information for both interaction with and translocation through the OM. Preliminary experiments with truncated CCHL proteins corroborate this result. The putative import signal must be contained within 150 central amino acids, since 80 N-terminal and 130 C-terminal amino acids can be deleted from CCHL without any influence on the import efficiency (G. Kispal, R. Lill and W. Neupert, unpublished results). Further experiments are necessary to define the precise location and the chemical character of the import signal in CCHL.

Aside from describing a novel import pathway into the IMS, our studies on CCHL import bear relevance to the general protein import pathway into mitochondria. Import of proteins across the OM is not necessarily coupled to translocation across the IM, i.e. the two translocation machineries in the two membranes do not necessarily form a continuous channel. Indication for this has come already from studies on translocation intermediates in the IMS en route to the matrix. Segments of the polypeptide chains in transit are accessible to added protease after rupture of the OM indicating that the intermediates are exposed to the IMS [40,41]. Sequential transfer across the OM and IM in intact mitochondria was observed for CCHL fusion proteins carrying a matrix targeting sequence at the N-terminus (Fig. 2B). Without a membrane potential, the fusion proteins were imported into the IMS like CCHL itself. Upon establishing a membrane potential, at least the N-terminus of the proteins was transferred from the IMS to the matrix as indicated by the cleavage of the signal sequence (G. Kispal, R. Lill and W. Neupert, unpublished results). Taken together, these data suggest that transport across the OM and IM can occur sequentially by using two principally independent translocation machineries [17,42]. Contact sites between the OM and IM may then be considered as dynamic structures that kinetically accelerate import across both membranes and thus may represent the structure that is predominantly used *in vivo* for the import of proteins into the matrix.

5. Perspectives

Future work on mitochondrial protein import has to concentrate on questions in two major directions. First, the as yet unknown components of the mitochondrial protein sorting apparatus have to be identified. Most importantly, these are the constituents of the import and export machineries in the IM. Furthermore, transit through and folding within the IMS may be mediated by still unidentified factors.

Drawing the analogy to the bacterial system, the participation of other chaperones in protein folding and assembly into oligomeric structures may be anticipated, e.g., activities like that of GroES or DnaJ homologs (see Chapters 25 and 26). Finally, other catalysts of post-translational modifications like new proteolytic processing activities may be discovered.

The second question is how the known components function and cooperate to finally achieve the correct localization and assembly of incoming proteins. This is a major challenge, but the discovery that translocation through the two membranes may be considered as two independent steps, will eventually allow to individually study the transit through the two membranes. It is thus easy to predict that future research on mitochondrial protein targeting and sorting will be as exciting as it has been in the past.

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