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Rosemary A. Stuart
Marquette University, rosemary.stuart@marquette.edu

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Insertion of Proteins into The Inner Membrane Of Mitochondria: The Role of The Oxa1 Complex

Rosemary A. Stuart
Department of Biology, Marquette University, 530 N. 15th Street, Milwaukee, WI

Abstract

The inner mitochondrial membrane harbors a large number of proteins that display a wide range of topological arrangements. The majority of these proteins are encoded in the cell's nucleus, but a few polytopic proteins, all subunits of respiratory chain complexes are encoded by the mitochondrial genome. A number of distinct sorting mechanisms exist to direct these proteins into the mitochondrial inner membrane. One of these pathways involves the export of proteins from the matrix into the inner membrane and is used by both proteins synthesized within the mitochondria, as well as by a subset of nuclear encoded proteins. Prior to embarking on the export pathway, nuclear encoded proteins using this sorting route are initially imported into the mitochondrial matrix from the cytosol, their site of synthesis. Protein export from the matrix into the inner membrane bears similarities to Sec-independent protein export in bacteria and requires the function of the Oxa1 protein. Oxa1 is a component of a general protein insertion site in yeast mitochondrial inner membrane used by both nuclear and mitochondrial DNA encoded proteins. Oxa1 is a member of the conserved Oxa1/YidC/Alb3 protein family found throughout prokaryotes throughout eukaryotes (where it is found in

mitochondria and chloroplasts). The evidence to demonstrate that the Oxa1/YidC/Alb3 protein family represents a novel evolutionarily conserved membrane insertion machinery is reviewed here.

Keywords

Oxa1, Yeast, Mitochondria, Protein sorting, Protein insertion, Oxa1/YidC/Alb3

1. Introduction

The mitochondrion, an eukaryotic organelle, harbors many protein complexes which function in metabolic pathways essential for the cell. The most notable of these is the aerobic production of energy in the form of ATP by a process termed oxidative phosphorylation. Mitochondria contains two membranes, an outer and an inner membrane, and two aqueous compartments, the mitochondrial matrix, which is enclosed by the inner membrane and a space located between the outer and inner membranes, termed the intermembrane space. Each of these compartments contains their own specific subset of mitochondrial proteins. The inner membrane is a protein-rich membrane, harboring many multi-subunit protein complexes involved in processes such as substrate and nucleotide transport, oxidative phosphorylation and proteolysis. The proteins residing in the inner membrane are coded for by genes located in either the nucleus or in the mitochondrial genome. Consequently, these proteins are either synthesized in the cell's cytoplasm or in the mitochondrial translational apparatus located in the matrix space. Prior to assembly, these proteins must be sorted from their sites of synthesis to become inserted into the inner mitochondrial membrane.

Research over the past years has indicated that a number of distinct pathways exist to sort the nuclear and mitochondrially encoded proteins to the inner membrane. The main focus of the review shall be devoted to the recent characterization of one of these pathways. This pathway, termed the Oxa1 pathway, involves export of proteins from the mitochondrial matrix into the inner membrane and is used by the mitochondrially encoded membrane proteins as well as a subset of nuclear encoded proteins.

2. Import of nuclear encoded proteins into mitochondria

The nuclear encoded proteins located in the inner membrane mitochondria are synthesized as precursor proteins in the cell's cytoplasm. These proteins are targeted to and become imported into the mitochondria, a process thought to occur in a post-translational manner [1], [2], [3], [4], [5], [6]. Nuclear encoded mitochondrial proteins contain targeting sequences, which ensure their initial targeting to the mitochondria. For many of these proteins, these mitochondrial targeting sequences are located at the amino-(N)-terminus of the polypeptide, and they become proteolytically removed by the mitochondrial processing peptidase (MPP) during or following import into the mitochondria [1], [2], [3], [4], [5], [6]. In some instances, however, proteins destined for the inner membrane contain internal, non-cleavable mitochondrial targeting sequences [4], [5], [6].

Import of nuclear encoded proteins to the mitochondrial inner membrane requires initial translocation across the outer mitochondrial membrane, a step mediated by a general import machinery located in the outer membrane, the translocase of the outer membrane (TOM) complex [1], [2], [3] (Fig. 1). Passage across, or insertion into, the inner membrane requires an energized inner membrane and is facilitated by another proteinaceous machinery, the translocase of the inner membrane (TIM) complex. Two TIM translocases differing in substrate specificity, the Tim17–23 complex [4], [5], [6] and the Tim22–54 machinery have been identified [7], [8]. The Tim17–23 complex appears to represent the general import machinery and is used by the presequence-targeted precursor proteins [4], [5], [6] (Fig. 1). Translocation of segments of preproteins across the inner membrane via this complex requires the co-operation of another component, Tim44 and the ATP-driven molecular chaperone mt-Hsp70 and its co-chaperone Mge1 [4], [5], [6]. The Tim22–54 complex mediates

the membrane insertion of a subset of polytopic inner membrane proteins, such as members of the metabolite carrier family and is reviewed by R.E. Jensen and C.D. Dunn in this issue [7], [8].

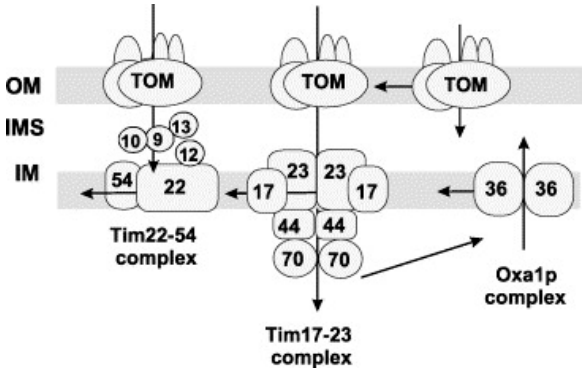

Fig. 1. Model of the protein translocases of the outer and inner membranes involved in the import and sorting of proteins in mitochondria. See text for details. Abbreviations: OM, outer membrane; TOM, translocase of the outer membrane; IM, inner membrane; TIM, translocase of the inner membrane.

Following the import into the mitochondria, precursor proteins can undergo a number of further maturation events, including proteolytic removal of mitochondrial targeting sequences, sorting to the inner membrane, folding and assembly into their functional oligomeric structures.

3. Proteins encoded by the mitochondrial genome

Although the majority of the proteins which make up these complexes embedded in the inner membrane are encoded by genes in the cell nucleus, a small percentage of the proteins (seven in the yeast *Saccharomyces cerevisiae*), are encoded by the mitochondrial genome located in the matrix compartment. These seven proteins are integral inner membrane proteins, all subunits of the respiratory chain complexes. These proteins are, cytochrome *b*, of the cytochrome bc_1 complex, subunits 1, 2 and 3 of the cytochrome oxidase complex (Cox1, Cox2 and Cox3 proteins, respectively) and F_1F_o-ATP synthase subunits, ATPase 6, ATPase 8 and ATPase 9. As outlined in more detail below, current evidence indicates that these mitochondrial gene products are inserted into the inner membrane as they are undergoing translation on the mitochondrial ribosomes. Each of these proteins represents essential subunits of their respective respiratory chain complexes. Failure of these proteins to reach the inner membrane results in a respiratory deficient phenotype for the yeast. Assembly of these multi-subunit respiratory chain complexes thus involves the coming together of proteins encoded by the nuclear and mitochondrial genomes in a coordinated fashion and with a precise stoichiometry.

4. Topogenic signals

Integral inner membrane proteins display a wide variation in their membrane topologies, differing with respect to the number of times they span the inner membrane and also in the location of their N- and C-termini (intermembrane space-exposed, “out” or matrix-exposed, “in”). Some proteins are anchored to the membrane by one membrane spanning or transmembrane segments (monotopic), while others span the inner membrane multiple times (polytopic proteins). All inner membrane proteins contain sequence determinants, so-called topogenic signals, which ensure the sorting of the protein to the inner membrane and which determine the attainment of their orientation in the membrane. These signals generally comprise hydrophobic cores (approximately 17–22 amino acids) flanked on both sides by charged amino acid residues [9]. For nuclear encoded inner membrane proteins, the topogenic signal, although distinct from the mitochondrial targeting signals, may operate in conjunction with the mitochondrial targeting information, to ensure sorting of the protein directly into the inner membrane while traversing the TIM machinery. In most cases, the topogenic

signals comprise an integral part of the sorted protein, i.e. the transmembrane segment. In a few cases, however, they are proteolytically removed by a peptidase (the Imp1/Imp2 complex) in the intermembrane space [10], [11], following sorting of the protein to the correct orientation in the membrane.

5. Pathways of protein sorting to the inner membrane

Bearing in mind the wide variety of final orientations of inner membrane proteins, together with their two possible sites of synthesis (cytoplasm for the nuclear encoded, and mitochondrial matrix for the mitochondrially encoded proteins), one mechanism alone cannot account for the mode of operation of all topogenic signals. Research from a number of laboratories over the past years has confirmed that a number of different sorting pathways may exist [4], [5], [6], [7], [8], [9].

5.1. Sorting by translocation arrest

The topogenic signals of some nuclear encoded inner membrane proteins function to arrest their translocation during import, at the level of the inner membrane, the essence of the “stop transfer” model [12], [13], [14], [15], [16], [17], [18]. Proteins using this pathway, following initiation of translocation across one of the two protein translocation machineries of the inner membrane (either the Tim17–23 or Tim22–54 complex) to become directly sorted to the inner membrane, laterally from the TIM complex (Fig. 2). Presequence-targeted proteins sorted to the inner membrane by a “stop-transfer” mechanism initiate import via the Tim17–23 machinery, where they are thought to become arrested and undergo a lateral transfer directly into the inner membrane (Fig. 2) [4], [9], [17], [18].

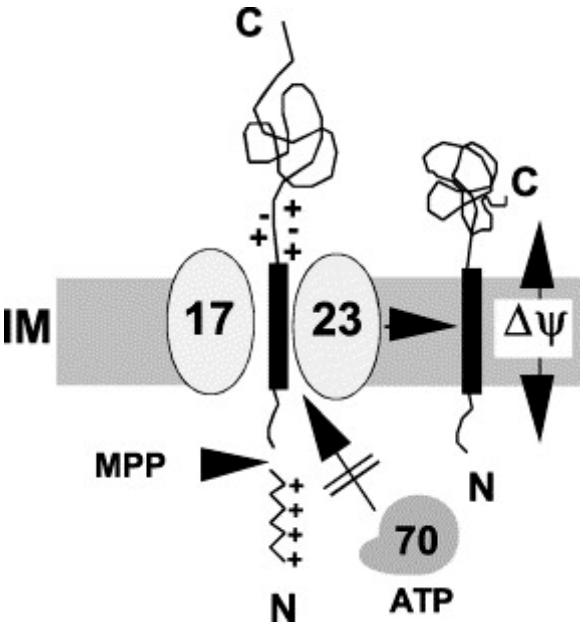

Fig. 2. Sorting of presequence-targeted proteins by translocation arrest during import. See text for details. Abbreviations, MPP, mitochondrial processing peptidase; IM, inner membrane.

Some proteins bearing internal mitochondrial targeting signals also use a translocation arrest and direct membrane insertion mechanism to reach the inner membrane. This group of proteins includes members of the carrier family, such as the ADP/ATP translocator, which employs the Tim22–54 translocase for membrane insertion [5], [6], [7], [8]. The Tim22–54 translocase functions closely with a number of small soluble proteins, Tim8, 9, 10, 12 and 13, resident proteins of the intermembrane space to mediate the import and insertion of its substrate proteins (Fig. 1) (see accompanying review by Jensen et al.) [5], [6], [7]. This pathway clearly differs

from that used by N-terminal targeted proteins, which are sorted by translocation arrest in the Tim17–23 machinery.

5.2. Sorting by export from the mitochondrial matrix

Nuclearly encoded polytopic inner membrane proteins can be divided into two classes, those that do not have prokaryotic counterparts and those that do. Proteins belonging to the first class include proteins such as the members of the carrier family, e.g. the ADP/ATP carrier family and components of the TIM machineries. As mentioned earlier, all available evidence suggests that these proteins become directly inserted into the inner membrane following a translocation arrest mechanism during import, in a manner facilitated by the TIM machinery [4], [5], [6], [7]. On the other hand, members of the second class of nuclearly encoded proteins, i.e. those which have prokaryotic equivalents and which are targeted to the mitochondria by N-terminal mitochondrial targeting signals are sorted by a very different mechanism. Proteins following this second route do not become arrested during import over the inner membrane, but rather continue into the matrix, from where they insert into the inner membrane via an independent export step [9], [19], [20], [21].

Proteins sorted to the inner membrane via an export pathway from the matrix display a diverse range of topologies and include both nuclear as well as mitochondrially encoded proteins. Sorting of proteins by export from the matrix was initially described for subset of inner membrane proteins, which are oriented in the membrane in such a manner that their N-termini are exposed to the intermembrane space (Fig. 3) [19], [20], [21], [22]. Examples of these proteins include subunit 9 (Su9) of the F_1F_o-ATP synthase, subunit 2 of the mitochondrially encoded cytochrome oxidase complex (Cox2) and a polytopic nuclear encoded protein, Oxa1 (Fig. 3).

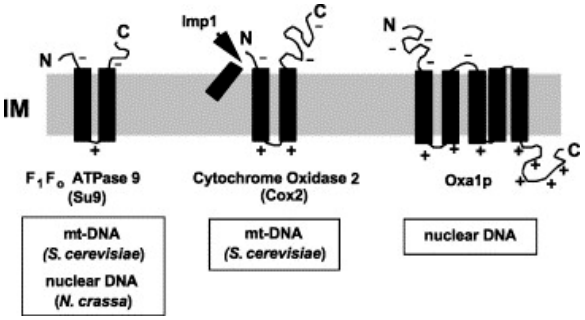

Fig. 3. Model of inner membrane proteins, ATPase 9, Cox2 and Oxa1, used in the initial analysis of mitochondrial protein export. The genomes on which these proteins are encoded are indicated in boxes below each protein. Each protein has a N_{out}-orientation in the inner membrane and in each case, the hydrophilic regions exposed to the matrix bear a net positive charge (+). The intermembrane space exposed segments bear either a net negative charge (−) or are neutral. Abbreviations, IM, inner membrane; Imp1, inner membrane peptidase 1; mt-DNA, mitochondrial DNA; *S. cerevisiae*, *Saccharomyces cerevisiae*; *N. crassa*, *Neurospora crassa*.

For nuclear encoded proteins sorted to the inner membrane along this export pathway, the steps of protein import and export are two distinct processes, which can be experimentally dissected. These proteins are first completely imported over the Tim17–23 machinery into the mitochondrial matrix where their N-terminal targeting presequences are proteolytically removed. Following maturation, these proteins embark a second independent membrane insertion/export step to become inserted into the inner membrane [9], [19], [20], [21], [23]. All the nuclear encoded proteins sorted in this manner, which have been characterized to date, achieve a final membrane orientation where their N-terminal regions are located in the intermembrane space. Hence, the membrane insertion of these proteins from the matrix is coupled to the export of N-terminal hydrophilic regions across the membrane to the intermembrane space. Accordingly, this translocation event has been referred to as “N-terminal tail export” [19], [20], [21], [23]. The process of N-

terminal tail export bears resemblance to the previously characterized process of N-terminal tail export in prokaryotes (see below) [9], [19], [23], [24], [25].

Export of the N-terminal tail of the nuclear encoded proteins from the matrix to the intermembrane space requires an energized inner membrane. Dissipation of the membrane potential (following post-translational import into the mitochondrial matrix) resulted in the accumulation of the imported proteins in the mitochondrial matrix [19], [20], [21], [23]. As will be outlined in detail below, insertion of these N-terminal tail exported proteins into the inner membrane is not a spontaneous event, but rather is facilitated by a protein complex in the inner membrane, termed Oxa1.

The process of protein export from the matrix was first demonstrated for presequence-targeted inner membrane proteins, which were initially imported into the mitochondrial matrix in a post-translational fashion [19], [20], [21]. It was then subsequently shown that mitochondrial gene products synthesized within the matrix also undergo a similar insertion-export step to attain their orientation in the inner membrane [20], [22]. Membrane insertion of the transmembrane segments of these proteins, like that of the nuclear encoded ones, is also coupled to the export of hydrophilic segments of the proteins completely across the membrane to the intermembrane space. Of all the mitochondrial gene products, the insertion of subunit 2 of the cytochrome oxidase complex (Cox2) probably represents the best-characterized pathway [20], [22], [26], [27] (Fig. 4).

Cox2 is an integral membrane protein, which spans the inner membrane twice in an N_{out}–C_{out}orientation. Attainment of this topology hence requires the insertion of two transmembrane segments and is coupled to the export of hydrophilic, negatively charged N- and C-termini. Cox2 is synthesized in the mitochondrial matrix as a precursor protein with an N-terminal cleavable presequence (pCox2) (Fig. 4). Insertion of pCox2 to a final N_{out}–C_{out} orientation was observed to be dependent on the presence of an energized inner membrane [20], [22]. In particular, export of the carboxy-(C)-terminal region, which is relatively long (approximately 150 amino acid residues) and bears a strong net negative charge, displayed a stringent requirement for a membrane potential across the inner membrane [20], [22]. Following export, pCox2 associates with a membrane-bound chaperone, Cox20 [28]. Association with Cox20 is necessary for proteolytic removal of the N-terminal presequence of pCox2, which results in the generation of the mature size Cox2 species [28]. This processing event is catalyzed by the Imp1/Imp2 protease located at the outer surface of the inner membrane [10], [11]. Current evidence suggests that Cox2, like the other mitochondrial gene products destined for the inner membrane, become inserted into the membrane as they are undergoing synthesis on mitochondrial ribosomes, i.e. in a co-translational fashion [26], [29], [30]. The insertion of Cox2 and export of its N- and C-termini is not a spontaneous event, but rather is facilitated by the Oxa1 protein complex located in the inner membrane [22], [27].

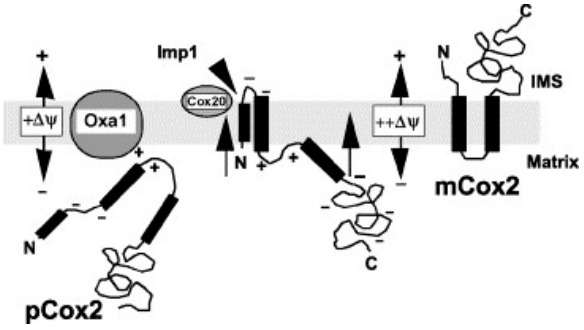

Fig. 4. Model of the export pathway of the mitochondrially encoded protein cytochrome oxidase subunit 2 (Cox2) following synthesis as a precursor form (pCox2) in the matrix. See text for details. Note for simplicity, the pCox2 species, prior to export, is depicted as a fully synthesized polypeptide in the mitochondrial matrix. As

outlined in the text, current evidence suggest that pCox2 is inserted into the inner membrane at the Oxa1 site in a co-translational manner. Abbreviations see Fig. 1.

6. The Oxa1 protein

The Oxa1 protein was initially identified in two independent genetic screens designed to identify yeast proteins involved in the biogenesis of the mitochondrial respiratory chain complexes [31], [32]. Characterization of a respiratory deficient mutant lacking cytochrome oxidase activity lead to the cloning of a nuclear gene termed OXA1, for *ox*idase *a*ssembly [31], [32], [33]. Oxa1 is a 36 kDa protein located in the mitochondrial inner membrane, where it spans the inner membrane five times in a N_{out}–C_{in} orientation (Fig. 3) [21], [31], [32], [33]. Sequence analysis of the Oxa1 protein indicated that it represented a highly conserved protein, present throughout both the eukaryotic and prokaryotic kingdoms (see below) [31], [32], [33], [34], [35], [36], [37].

Further characterization of *oxa1* mutants indicated that the observed respiratory deficiency was not caused by a lack of cytochrome oxidase assembly alone, but rather was also due to compromised F_1F_o-ATP synthase and cytochrome bc_1 complex activities [31], [32], [33], [38], [39], [40]. The pleiotrophic phenotype displayed by *oxa1* mutants was indicative of a general function of Oxa1 in the biogenesis of inner membrane proteins.

The first hint that Oxa1 may be involved in mediating the insertion of proteins from the matrix into the inner membrane came from an observation made with a temperature-sensitive yeast mutant of Oxa1, *pet ts1402*, which had been isolated by Bauer et al. [32]. When exposed to the non-permissive temperature of 37 °C, an accumulation of the precursor form of cytochrome oxidase subunit 2 (pCox2) was detected in this mutant [32]. As outlined previously, the precursor of Cox2 undergoes maturation by the Imp1/Imp2 peptidase in the intermembrane space, following its membrane potential-dependent export to an N_{out}–C_{out} topology (Fig. 4) [20], [22]. Thus, accumulation of the pCox2 species in this mutant at the non-permissive temperature reflected either lack of correct membrane insertion/export or the lack of Imp1/Imp2 activity. Using mitochondria isolated from the *oxa1* mutant, it could be shown that Oxa1 plays an essential role in the sorting of newly synthesized pCox2 from the matrix to the intermembrane space, rather than in the subsequent event of proteolytic maturation [27]. The phenotype of the *pet ts1402* mutant was selectively induced in vitro, by briefly exposing mitochondria, which had been isolated from this strain grown at permissive temperature, to the elevated non-permissive temperature of 37 °C [27]. Following induction of the phenotype in the isolated mitochondria in this manner, newly synthesized pCox2 accumulated in the matrix as it failed to undergo export to an N_{out}–C_{out} topology [27]. In an independent study performed by He and Fox [22], the export of Cox2 and Cox2-derivatives was found to be inhibited in *oxa1* null mutant in vivo.

In addition to playing an essential role in the biogenesis of Cox2, Oxa1 was subsequently shown to be required for the insertion of those nuclear encoded proteins, including Oxa1 itself, which undergo N-tail export and insertion into the inner membrane, following prior import into mitochondrial matrix [22], [26], [27], [28], [29].

In summary, the Oxa1 complex acts as a general insertion site mediating the insertion of both nuclear and mitochondrially encoded proteins from the mitochondrial matrix into the mitochondrial inner membrane.

7. Oxa1 function is not limited to the insertion of N-tail exported proteins

The function of Oxa1 was initially demonstrated to be directly required for the export of at least the N-terminal tail region of these membrane proteins [26], [27]. Export of other regions of membrane proteins to the intermembrane space, such as hydrophilic loops between neighboring transmembrane domains as well as C-terminal hydrophilic segments were also inhibited in *oxa1* mutant mitochondria [22], [26], [27], [28]. Export of the N-terminal tails of these proteins from the matrix was found to kinetically precede and even be a prerequisite for, the export of internal and C-terminal hydrophilic regions of the proteins,

however [20], [21], [26]. Thus, from these initial studies, it could not be determined whether the export of domains, other than the N-terminal regions, directly required the function of the Oxa1 complex or not [26].

The function of Oxa1 was recently shown not to be limited to the insertion of N-tail exported membrane proteins, but rather it extends to the insertion of polytopic proteins bearing N-termini that are retained in the mitochondrial matrix. Mitochondrial gene products, subunit 1 (Cox1) and subunit 3 (Cox3) of the cytochrome oxidase complex and cytochrome *b* of the cytochrome bc_1 complex, span the inner membrane several times (12, 7 and 8 times, respectively), in a manner where their N-termini reside in the mitochondrial matrix [41], [42]. Attainment of this topology thus involves the translocation of hydrophilic loops between neighboring transmembrane segments, across the inner membrane, to their final destination in the intermembrane space. Unlike the export of Cox2, the insertion of these other proteins did not display a strict requirement for the presence of an energized inner membrane [29], [43]. As was initially demonstrated for Cox2, the Oxa1 complex also supports the insertion of other mitochondrially encoded proteins into the inner membrane [28]. Using mitochondria isolated from the temperature-sensitive *oxa1* mutant, newly synthesized Cox1, Cox3 and cytochrome *b* were shown to display a dependency on Oxa1 function for their efficient integration into the inner membrane. Thus, it was concluded that the function of Oxa1 was not to be limited to the export of N-terminal tails of membrane proteins, but rather it represents a component of a general membrane insertion site for a class of proteins of the mitochondrial inner membrane [29].

8. Oxa1 directly facilitates membrane insertion of newly synthesized proteins

From the studies described above, it became evident that the function of Oxa1 was required for the insertion of a variety of proteins, both nuclear and mitochondrially encoded, into the inner membrane. Using a chemical cross-linking approach, it could be demonstrated that Oxa1 directly interacts with substrate proteins as they are undergoing the insertion step into the inner membrane [26], [29]. Nascent polypeptide chains being synthesized in mitochondria, as well as newly imported nuclear encoded proteins, were shown to physically interact directly with Oxa1, as they were undergoing export from the matrix [26]. Of the mitochondrial gene products, newly synthesized Cox1, Cox2, Cox3 and cytochrome *b* were found to physically interact with Oxa1 [26], [29]. Although the Oxa1-mediated insertion of Cox2 into the membrane displays a strong dependency on the presence of membrane potential, the interaction of newly synthesized Cox2 with Oxa1 occurred even in the absence of an energized membrane [29]. Thus the interaction of Oxa1 with Cox2 and possibly other membrane proteins occurs early and independently of membrane insertion. In summary, based on their dependency on the presence of Oxa1 for insertion and the observed physical interaction of Oxa1 with its substrates, it was concluded that Oxa1 was directly required for the membrane insertion of these proteins into the inner membrane [26], [29].

The mitochondrially encoded proteins were shown to interact with Oxa1 early during their biogenesis, i.e. during their synthesis as nascent polypeptide chains [26], [29]. Cross-linking of full-length polypeptide chains to Oxa1 was obtained only when the cross-linking was performed during ongoing protein synthesis. If completion of protein synthesis was blocked by the addition of chloramphenicol prior to cross-linking, nascent chains, rather than full-length proteins, were found in association with Oxa1 [29]. Thus a nascent polypeptide chain could be cross-linked to Oxa1 prior to, but independent of, the completion of its synthesis. Moreover, the association of Oxa1 with the nascent chain undergoing membrane insertion was enhanced by the presence of the associated ribosome. Release of the ribosome by the addition of puromycin (puromycin causes premature release of the nascent chain from the ribosome) prior to cross-linking, resulted in a strong decrease of the level of nascent polypeptide which remained in physical proximity to Oxa1 [29].

In summary, Oxa1 interacts directly with the nascent chain as it is undergoing membrane insertion. This interaction is supported by the presence of the associated ribosome (Fig. 5). Mitochondrial ribosomes actively undergoing translation have been observed to be in close proximity to the mitochondrial inner membrane in

vivo [44], [45]. A close coupling of the synthesis of the membrane proteins encoded by the mitochondrial DNA and their translocation into the membrane has been suggested to occur [30], [46], [47]. Consequently, mitochondrial gene products destined for the inner membrane are synthesized directly at their site of membrane insertion, the Oxa1 complex.

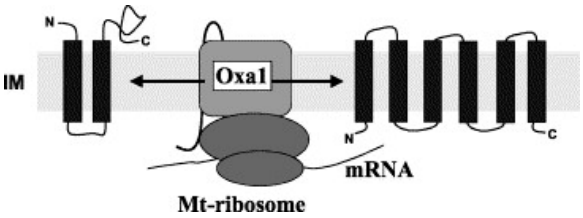

Fig. 5. Insertion of mitochondrially encoded polytopic proteins into the inner membrane, mitochondrial ribosome activity undergoing protein translation, are closely associated with the mitochondrial inner membrane. Nascent peptides physically associate with Oxa1 as they are undergoing insertion. Current evidence suggests that a component(s) of the translating ribosome may bind directly to the Oxa1 complex. See text for more details. Abbreviations, Mt-ribosome, mitochondrial ribosome; IM, inner membrane.

A close coupling of the translation machinery with the Oxa1 insertion site in this manner may serve two purposes. First, coupling of translation and translocation events would circumvent the possibility of these hydrophobic proteins being synthesized in the matrix prior to their membrane insertion, where they may aggregate. Second, a co-translational insertion event may be energetically favorable by providing a driving force required for the insertion and translocation events.

How are mitochondrial ribosomes undergoing translation targeted to the Oxa1 complex? How is this interaction of the translating ribosome with the Oxa1 complex maintained? The molecular basis for such an interaction may be complex in nature and would also need to ensure that the translating ribosome could be distinguished from one not undergoing translation. Binding of the translating ribosome to the Oxa1 complex may involve a direct interaction of Oxa1 with component(s) of the translating ribosomes, such as a ribosomal subunit, the mRNA being translated and/or the nascent chain emerging from the ribosome. Interestingly, Fox et al. [30], [46], [47] have genetically identified a number of translational activator proteins, which are integral inner membrane proteins. By interacting with the 5′ untranslated leaders of specific mRNAs, Fox [30], [46], [47], [48] has proposed that these membrane embedded translational activators may serve to ensure that initiation of synthesis of the membrane proteins occurs directly at the level of the inner membrane. It is therefore plausible that such translational activator proteins may closely cooperate with the Oxa1 complex to facilitate the recognition and binding of ribosomes and their associated mRNAs. By doing so, both the initiation and the completion of synthesis of these membrane proteins would occur in the environment of the Oxa1-membrane insertion site.

It is possible that ribosomes undergoing translation at the inner membrane surface are directly in contact with Oxa1 or components of the Oxa1 complex. The matrix-exposed hydrophilic regions of Oxa1, in particular the C-terminal segment (approximately 100 residues in length and basic in nature) may be involved in recognizing and/or binding component(s) of the translating ribosome. Further characterization of the Oxa1 complex is required to elucidate the molecular details of how co-translational insertion of proteins into the membrane is ensured.

9. Does the Oxa1 complex represent the only membrane insertion site for proteins being exported from the matrix?

Oxa1 represents a component of the general protein insertion machinery involved in the insertion of a variety of inner membrane proteins differing both in their membrane orientation and in their site of synthesis. A number of independent experimental observations would support that the Oxa1 complex, however, may not be the only site for insertion of proteins into the inner membrane from the matrix. Increasing evidence would indicate that the level of dependency on Oxa1 for membrane insertion varies among different substrate proteins [26], [29]. Of all the substrates tested, the mitochondrially encoded Cox2 protein displayed the strictest dependency on Oxa1 for membrane insertion [22], [27]. In contrast, when investigated in parallel, the insertion of other newly synthesized mitochondrially encoded proteins studied, in particular cytochrome *b*, Cox3 and ATPase 6, was only partially impaired in the *oxa1* temperature-sensitive mutant mitochondria [29]. In addition, although the Oxa1 complex was shown to be required for the membrane insertion from the matrix of newly imported Oxa1 precursor protein, in contrast to pCox2, the inhibition of Oxa1 insertion in the *oxa1* mutant was not complete [26]. This observation is indicative of the presence of an alternative insertion activity in the inner membrane. This notion is reinforced when one considers that the *oxa1* null mutant can be rescued by expression of the Oxa1 from a plasmid-borne gene [31], [34], [35]. If the presence of Oxa1 in the mitochondrial membrane was obligatory for membrane insertion, the *oxa1* null mutant should not have been able to be complemented in this manner. Although it cannot be excluded that some proteins may undergo a limited spontaneous insertion into the inner membrane, taken together, these observations may be indicative of an alternative Oxa1-independent insertion process which displays partial overlapping specificity with the Oxa1 machinery.

Further evidence for the existence of an alternative Oxa1-independent insertion process stems from the elegant work of Dujardin et al. [49], [50]. Taking a genetic approach to understanding the function of Oxa1, Dujardin reported that mutations in the transmembrane region of cytochrome c_1 restored growth of the *oxa1* null mutant on non-fermentable carbon sources [49]. Although the molecular basis for this intriguing observation is not yet understood, the finding further illustrates that the inner membrane displays significant flexibility to facilitate insertion of its resident proteins. In a different study, Dujardin et al. [50] showed that deletion of the gene encoding Yme1, an inner membrane protease in the *oxa1* null mutant background, resulted in the restoration of the F_1F_o-ATP synthase to wild-type levels. Thus, under conditions where the Yme1-catalyzed proteolysis of non-assembled subunits of the F_o-ATP synthase was prevented, the efficiency of the Oxa1-independent insertion step of F_o-ATP synthase subunits was apparently sufficient to ensure assembly of a functional ATP synthase [50].

What is the molecular basis for the Oxa1-independent insertion pathway(s)? Evidence that another protein, Mba1 may represent and Oxa1-independent insertion site was recently presented [51]. Mba1 was originally identified as a multicopy suppressor of a yeast mutant lacking either the Yta10 (Afg3) or Yta12 (RCA1) proteases in the mitochondrial inner membrane [52], [53]. Interestingly, this screen also identified Oxa1 as a suppressor of the *yta10*(*afg3*) and *yta12*(*rca1*) null mutant strains [52], [53]. Like Oxa1, the presence of Mba1 was shown to influence the efficiency of insertion of a number of mitochondrially encoded inner membrane proteins [51]. Furthermore, Mba1, like Oxa1, interacts directly with newly synthesized proteins, which become inserted into the inner membrane. Although the function and substrate specificity of Oxa1 and Mba1 appear to overlap, available genetic and biochemical data would suggest that Mba1 is a component of an alternative, Oxa1-independent insertion site for membrane proteins [51].

Other candidate components for such an alternative Oxa1-independent insertion mechanism may also involve the inner membrane proteins Pnt1 or Cox18. Pnt1 was identified in a genetic screen designed to identify proteins involved in the export of Cox2 and derivatives [54]. Cox18, a protein required for the assembly of the

cytochrome oxidase, shares some sequence similarity with Oxa1 [55]. Further characterization of both of these proteins is required to fully elucidate their possible involvement in the membrane insertion process (see Note Added in Proof).

10. Oxa1 is a member of a novel family of evolutionarily conserved proteins involved in the membrane insertion of proteins

According to the endosymbiont hypothesis, the inner membrane of mitochondria and the thylakoid membrane of chloroplasts are derived from the plasma membrane of their respective prokaryotic ancestors. Many of the mitochondrial inner membrane proteins are homologous to bacterial proteins thus reflecting their prokaryotic ancestry. Analysis of the charge-distribution profile of their hydrophilic segments on either side of the inner membrane suggested that these mitochondrial proteins adhere to the “positive-inside” rule of protein sorting, initially described for bacterial membrane proteins [56], [57], [58], [59]. This observation led to the prediction some years ago that these mitochondrial proteins are sorted to their inner membrane orientations along a prokaryotic type of export pathway [9], [56].

Further characterization of the Oxa1-mediated export pathway used by mitochondrial inner membrane proteins demonstrated it to resemble the bacterial Sec-independent membrane insertion pathway with respect to energetic requirements, adherence to the “positive-inside” rule and independence on the Sec translocation machinery [19], [20], [23]. In mitochondria, the involvement of a SecYEG-type of machinery was considered unlikely, as there is no indication, at least in yeast mitochondria, for the existence of a Sec machinery [60].

The most convincing evidence to demonstrate that the Oxa1-mediated export pathway in mitochondria was indeed conserved from the prokaryotic ancestors of mitochondria was revealed following the functional characterization of Oxa1 homologs from other organisms.

Oxa1 is an evolutionarily conserved protein found throughout prokaryotes and eukaryotes. In prokaryotes it is found in gram positive and gram negative bacteria, where it is known as YidC (in *Escherichia coli*) or the conserved 60 kDa inner membrane protein, IM60 [37], [61], [62]. In eukaryotes, both mitochondrial and chloroplast homologs have been reported [31], [32], [36]. The Oxa1 homolog in chloroplasts is termed Albino3 (Alb3) and it is an integral membrane protein of the thylakoid membrane system [36].

Following the elucidation of the role of Oxa1 in the mitochondrial protein export pathway, it was proposed that the non-mitochondrial Oxa1 homologs may perform a similar function as Oxa1, namely, in facilitating the insertion and possible translocation of protein segments into and across their respective membranes [22], [26]. Recently, evidence to confirm that the Oxa1/YidC/Alb3 proteins represent a conserved protein family mediating the insertion of proteins into their respective membrane system was presented [62], [63], [64].

The chloroplast homolog of Oxa1, Alb3 was identified in *Arabidopsis* a number of years ago [36]. Mutants failing to synthesize Alb3 displayed an albino phenotype, thus indicating this thylakoid membrane protein was required for thylakoid membrane biogenesis. Earlier work from a number of research laboratories had indicated that at least three distinct sorting pathways exist in chloroplasts to target proteins to the thylakoid membrane [65]. Recently, Alb3 was demonstrated to play a crucial role in the insertion of at least one membrane protein, the light harvesting chlorophyll-binding protein (LHCP) into the thylakoid membrane of chloroplast [63]. These results implicate the role of Alb3 in at least one of the sorting pathways used by the thylakoid membrane proteins and support the proposal that the non-mitochondrial homologs of Oxa1 are involved in facilitating protein translocation events [63].

In prokaryotes, the insertion into and translocation across the periplasmic membrane of many proteins is mediated by a general translocase, termed the SecYEG translocase (Sec-dependent transport) [66], [67], [68].

The insertion of a number of other membrane proteins, however, has been reported to occur in a Sec-independent manner [24], [25], [68]. Although it remained elusive for some time, the identity of this Sec-independent translocase in *E. coli*, was recently shown to be YidC, the Oxa1 homolog [62]. In addition to mediating Sec-independent translocation, YidC was also shown to physically interact with and support the insertion of Sec-dependent membrane proteins [62], [69], [70], [71], [72]. By physically interacting with the SecYEG translocase, YidC has been proposed to play a role in mediating the membrane insertion of hydrophobic signal sequences or transmembrane domains of protein which are undergoing translocation across the Sec machinery [70], [71], [72]. Thus, YidC may serve to enhance the lateral partitioning of membrane proteins into the periplasmic membrane.

The findings on the *E. coli* homolog of Oxa1, YidC, have important implications for the mechanistic details of the mode of the mitochondrial Oxa1 action. They also raise a number of important questions. Firstly, the observations imply that YidC may not operate alone as a translocase to facilitate the membrane insertion of proteins, but rather it can act in concert with the general protein translocation machinery. Is this mode of action conserved for the mitochondrial homolog of YidC, Oxa1? As there is no mitochondrial Sec machinery (at least in yeast) [60], does this mean that Oxa1 acts alone as a general protein translocase? Further research, including the isolation and functional reconstitution of Oxa1 complex, will be needed to answer these questions.

Thus YidC in bacteria, Alb3 in chloroplasts, and Oxa1 in mitochondria have similar functions; namely facilitating the insertion of proteins into biological membranes. Taken together, these findings demonstrate that the Sec-independent pathway of protein insertion into membranes has been conserved throughout evolution. This observation is in agreement with the endosymbiont hypothesis for the origin of mitochondria and chloroplasts. In summary, the Oxa1/YidC/Alb3 protein family represents a novel evolutionarily conserved membrane protein insertion machinery [29], [62], [64], [73].

Note Added in Proof

Since the preparation of this manuscript two important papers concerning Oxa1 and its role in the insertion membrane proteins have been published. The first is from Nargang and colleagues and deals with the characterization of the Oxa1 complex from *Neurospora crassa*. The authors report the purification of the Oxa1 complex and demonstrate it to be a homo-oligomeric complex, possibly consisting of four Oxa1 subunits [74]. Second, Fox and colleagues recently presented genetic and biochemical evidence for the involvement of three interacting membrane proteins, Cox18, Pnt1 and Mss2, in the export of the specifically the C-terminal region of Cox2 [75]. These findings suggest that the N-terminal (Oxa1-dependent) and C-terminal regions of Cox2, may be translocated across the inner membrane by genetically distinct protein complexes.

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