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Review

The multifaceted mitochondrial OXA insertase

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Most mitochondrial proteins are synthesized in the cytosol and transported into mitochondria by protein translocases. Yet, mitochondria contain their own genome and gene expression system, which generates proteins that are inserted in the inner membrane by the oxidase assembly (OXA) insertase. OXA contributes to targeting proteins from both genetic origins. Recent data provides insights into how OXA cooperates with the mitochondrial ribosome during synthesis of mitochondrial-encoded proteins. A picture of OXA emerges in which it coordinates insertion of OXPHOS core subunits and their assembly into protein complexes but also participates in the biogenesis of select imported proteins. These functions position the OXA as a multifunctional protein insertase that facilitates protein transport, assembly, and stability at the inner membrane.

Highlights

The oxidase assembly (OXA) insertase family represents an example of evolutive functional specialization.

OXA insertase facilitates the membrane integration of proteins from different genetic origins.

OXA association with mitochondrial ribosomes facilitates the insertion of newly synthesized mitochondrial-encoded proteins.

OXA links protein insertion to the early steps of oxidative phosphorylation biogenesis.

OXA1 facilitates protein translocation and assembly in mitochondria

Among eukaryotic cell organelles, mitochondria display a remarkable feature regarding their protein biogenesis resulting from their endosymbiotic origin. While most mitochondrial proteins are nuclear-encoded and imported from the cytosol, mitochondria contain their own genome (mtDNA) and a dedicated transcription and translation machinery [1–3]. To facilitate the import of proteins into mitochondria, cargo-specific protein translocases are required [4–8]. Yet, the mitochondria-encoded proteins also need to be inserted into the lipid phase of the inner mitochondrial membrane. This translocation process requires OXA1 insertase. While OXA1 is essential for the translocation of mitochondria-encoded proteins, it also contributes to the inner membrane insertion of some nuclear-encoded proteins. Here, we discuss the identification of OXA1, its functions, and interaction network partners that lead to the developing view of OXA as a module that acts as a platform for protein translation, membrane insertion, and protein assembly.

Chronicle of Oxa1 identification

OXA1 was identified in the yeast *Saccharomyces cerevisiae* as a conserved gene (*PET1402/OXA1*) important for respiratory growth, cytochrome *c* oxidase biogenesis, and export or assembly of the mitochondria-encoded Cox2 subunit [9,10]. OXA1 spans the inner mitochondrial membrane five times, exposing the C terminus into the matrix. Further studies on the *oxa1* mutant implicated Oxa1 in the cytochrome c oxidase assembly and the F_1F_O-ATP synthase [11]. Oxa1 is required to export N and C termini of Cox2 across the inner membrane but also interacts with a range of mitochondria-encoded nascent polypeptide chains [12]. Moreover, Oxa1 contributes to the translocation of nuclear-encoded imported proteins as the transport of the nuclear-encoded Oxa1 is defective in mutant mitochondria lacking Oxa1 [12]. The effect of a loss of Oxa1 on the cytochrome *c* oxidase differs from the impact on the F_1F_O-ATP synthase. Although reduction of the fully assembled F_1F_O-ATP synthase complex and its enzymatic activity were apparent in the absence of Oxa1, the biogenesis of the complex appeared not effectively blocked [11,13]. A post-translational interaction of Oxa1 with ATP9 indicated that Oxa1 contributes to the late steps of F_1F_O-ATP synthase assembly. Oxa1 was found to

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interact with the fully assembled F_1F_O-ATP synthase, suggesting that the Oxa1 insertase is involved in cotranslational processes and participates in assembly but also displays interaction with the oxidative phosphorylation (OXPHOS) system [13].

Mammalian OXA1 homolog – OXA1L

The human OXA1L shares 33% sequence identity with the yeast ortholog and complements the loss of Oxa1 in yeast [14]. Recently, a patient with affected OXA1L function was identified [15]. The patient displayed severe mitochondrial encephalopathy, developmental delay, hypotonia, and respiratory chain deficiency. This phenotype originated from heterozygous mutations in the *OXA1L* gene. One allele contained an 8 bp duplication, causing a frameshift (c.500_507dup, p.[Ser170Glnfs*18]), while the other showed a substitution c.620G>T inducing p.(Cys207Phe) exchange and affected the splicing so that exon 4 is skipped. Hence, the mature protein lacks an amino acid stretch (p.[Cys207_Glu254del]). Patient fibroblasts and skeletal muscle displayed reduced levels of complexes I, IV, and V [15]. Yet, under conditions of OXA1L knockdown, different effects on mitochondrial OXPHOS complexes were observed. Stiburek *et al.* found reduced complexes I and V levels in HEK293 cells with a stable knockdown of OXA1L [16]. Thompson *et al.* reported reduced amounts of complexes I and III–V, and the mitochondrial ribosome in inducible HEK 293T OXA1L knockout cells [15]. In summary, loss of OXA1L affects the human mitochondrial OXPHOS system, especially those complexes that contain mitochondria-encoded subunits. Accordingly, the severity of the observed effect appears to be linked to the magnitude of protein reduction and the analyzed cell type.

N- and C-terminal export

Together with Oxa1, the Oxa1-related Cox18 protein is required to export the mitochondria-encoded Cox2 [17]. In particular, yeast Cox18 is implicated in the export of the Cox2 C-terminal domain [18]. While Cox18 shares sequence similarities with Oxa1, it lacks the C-terminal matrix exposed ribosome binding domain of Oxa1 (see below) [19]. Both proteins fulfill distinct functions, indicated by the fact that overexpression of Oxa1 does not fully complement the loss of Cox18 [18,20]. Apparently, Cox18 is primarily involved in the Cox2 C-terminal export [19,21] while Oxa1 plays a role in both N- and C-terminal export of Cox2 [22], placing the two homologs into related yet distinct roles in mitochondrial protein export. Similar to Oxa1, Cox18 was shown to be functionally conserved among eukaryotes and identified as a transient interactor of COX2 in human cells [23,24].

Oxa2 was identified in *Neurospora crassa* as an Oxa1-related protein. Complementation studies in *S. cerevisiae* revealed a closer functional link to the yeast Cox18. The growth phenotype on nonfermentable media, Cox2 levels, and complex IV activity could be partially restored in *cox18* cells upon expressing $Oxa2^{NC}$. However, $Oxa2^{Nc}$ expression could not rescue the respiration defect in *oxa1* cells [25]. When studying the interaction of Oxa2 with newly synthesized proteins, co-immunoprecipitation with Cox1, Cox2, and Cox3 was observed, and pulse-chase experiments showed that the interaction of Cox2 and Cox3 with Oxa2 remained more stable than with Oxa1. These observations indicate a role for Cox18/Oxa2 downstream of the Oxa1 function [25].

OXA insertase family: import machinery across kingdoms

The bacterial plasma membrane, mitochondrial inner membrane, and chloroplast thylakoid membrane share related insertion machinery represented by the proteins YidC, Oxa1, and Alb3. A common feature of these proteins is the presence of five characteristic transmembrane domains. While the first two N-terminal hydrophobic stretches appear important for the insertase

activity and are conserved among eubacteria, mitochondria, and plastids, the remaining three C-terminal transmembrane regions are more variable [26]. Functional similarities between these proteins are supported by complementation studies on YidC by yeast Oxa1 and chloroplast Alb3 [27,28]. YidC is involved in two different protein insertion pathways: the Sec-translocon-dependent and Sec-translocon-independent pathways. In the first of these pathways, YidC interacts with the Sec-translocon during the insertion of membrane proteins and acts as the recipient of the nascent protein in proximity to the ribosomal exit site, thereby supporting the translocation of hydrophobic regions across the membrane [29]. Similarly, Oxa1 interacts with newly synthesized polypeptide chains and contributes to the early steps of their biogenesis [28,30]. The second YidC-dependent pathway, independent of the Sec-translocon, resembles the protein insertion process into the mitochondrial inner membrane from the matrix site. One prominent substrate of YidC in this pathway is the subunit c of the F_1F_O-ATP synthase [31]. In chloroplasts, Alb3 facilitates the insertion of the light-harvesting chlorophyll-binding protein subunits and is likely involved in the folding or assembly of cpSec61-inserted proteins [32].

While the YidC/Oxa1/Alb3 family was long thought to lack eukaryotic homologs that function outside endosymbiotic organelles, this family was recently expanded. Based on structural similarities, GET1, EMC3, and TMCO1, which act in the endoplasmic reticulum (ER), were shown to be related to the Oxa1 family [33,34]. Moreover, genetic analysis in which an Emc6–Emc3 fusion protein was targeted into yeast mitochondria showed that these proteins could partially restore Oxa1 function concerning protein insertion into the membrane; however, it did not support the assembly process of Atp9 [35]. Accordingly, the mechanisms by which these proteins facilitate protein insertion into the lipid phase are conserved. This raises the question of whether these similarities are based on evolutive functional diversification or convergent evolution depending on the biophysical proprieties of transported protein cargoes.

Protein import facilitated by Oxa1/OXA1L

Most information regarding Oxa1/OXA1L function is based on studies using yeast as a model system. While Oxa1 was initially established as a protein required for the biogenesis of mitochondria-encoded proteins (see above), Oxa1 also contributes to the biogenesis of select nuclear-encoded proteins such as Oxa1 itself [12,36,37]. This role of Oxa1/OXA1L in membrane insertion of nuclear-encoded proteins that are first translocated into mitochondrial is referred to as conservative insertion [36,38,39]. Such Oxa1-dependent proteins are mostly imported into mitochondria in a TIM23-dependent manner. Once these proteins are fully or partially imported into the matrix, Oxa1 facilitates inner membrane translocation. Considering the directionality of the transport process that resembles the topology of bacterial protein export and the relation of Oxa1 to YidC, the term conservative insertion was chosen to describe the process (Figure 1). The substrates that follow this sorting pathway are multispanning membrane proteins. One example is newly imported Oxa1, which is inserted into the membrane by Oxa1 [38,40]. Other examples are the ABC transporter Mdl1 and the succinate dehydrogenase subunit Sdh3, which are transported by the TIM23 complex. While TIM23 mediates membrane insertion of strongly hydrophobic transmembrane spans in a stop–transfer mechanism, less hydrophobic transmembrane spans are inserted by Oxa1 [36,37]. Therefore, it is tempting to speculate that an Oxa1-like (YidC-like) translocase was present in the prokaryotic ancestor of mitochondria that co-evolved with the appearance of the other translocases. Cox18 is a member of a phylogenetic tree branch of the Oxa1p/YidC/Alb3 protein family [25]. YidC topology is similar to Cox18 and can partially complement Cox18-deficient strains [41]. Yet, Cox18 is only dedicated to transport reactions of mitochondrial-encoded proteins. Moreover, Cox18 lacks the C-terminal matrix domain present in Oxa1 that enables interaction with the mitochondrial ribosome [25].

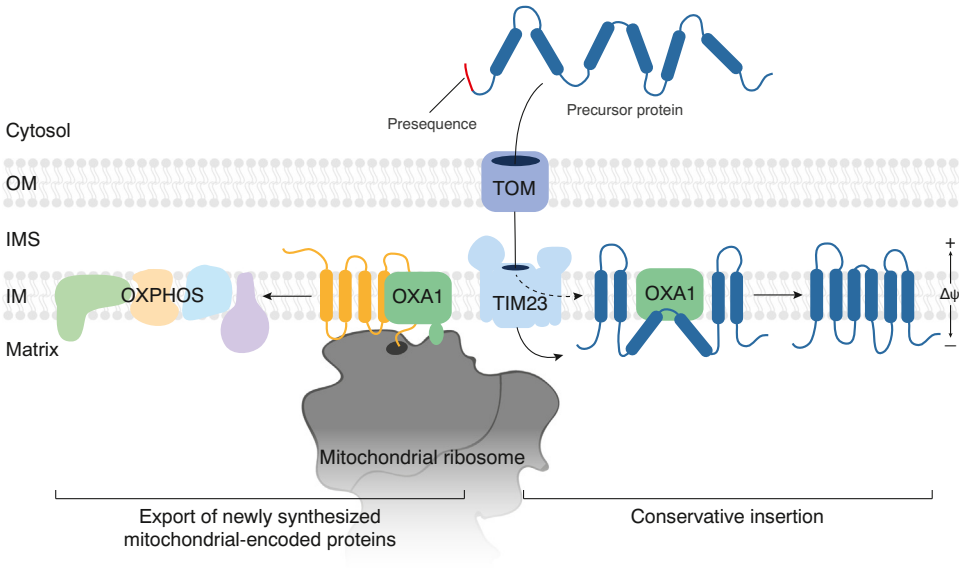

Figure 1. Involvement of oxidase assembly (OXA)1 family in protein translocation. Core components of the oxidative phosphorylation machinery (OXPHOS) are encoded on the mitochondrial genome and synthesized by mitochondrial ribosomes. The translating ribosomes associate with the membrane-embedded Oxa1/OXA1L, which inserts the newly synthesized proteins cotranslationally into the inner membrane (IM). Moreover, some presequence-carrying membrane proteins are translated by cytosolic ribosomes. They are transported across the outer mitochondrial membrane (OM) by the translocase of the outer membrane (TOM) and subsequently handed over to the presequence translocase of the inner membrane (TIM23). The protein can be partially released into the IM by TIM23-dependent stop–transfer or fully imported into the matrix. Finally, noninserted transmembrane spans are translocated or exported by Oxa1/OXA1L through conservative insertion. Both Oxa1 involving pathways, the export of mitochondrial encoded proteins, and the conservative insertion are membrane potential dependent. Abbreviation: IMS, intermembrane space.

Export of newly synthesized mitochondrial-encoded proteins

The prominent role of Oxa1/OXA1L is the insertion of newly synthesized mitochondrial-encoded proteins into the inner membrane (Figure 1). Oxa1 crosslinks to nascent chains of mitochondrial-encoded proteins [30]. Moreover, the C-terminal matrix-exposed domain of Oxa1 binds to the mitochondrial ribosome to enable the cotranslational insertion of nascent polypeptides into the inner membrane [42,43]. Yet, the paths of the newly synthesized polypeptides in mitochondrial ribosomes from *N. crassa* and *S. cerevisiae* are different [44]. The Oxa1–ribosome interaction is thought to occur through electrostatic forces between the positively charged C-terminal domain and the large ribosomal subunit and in a nascent-chain-independent manner [42,43]. This could be supported by Mba1, an inner mitochondrial membrane protein that serves as a second interaction site for the ribosomes [45,46]. Yet, the absence of the C-terminal Oxa1 domain leads to the accumulation of translation products in the matrix [42]. Interestingly, the mitochondrial ribosome only associates with the mammalian OXA1L when the ribosome is actively translating [47,48].

OXA involvement in OXPHOS complex assembly

The mammalian mitochondrial genome encodes 13 core subunits of the OXPHOS system. OXA1L mediates the cotranslational insertion of those newly synthesized proteins into the membrane but also forms complexes with membrane proteins that assist in the assembly process of the newly synthesized proteins, so-called assembly factors. Accordingly, the interaction between the newly synthesized polypeptide chain and OXA1L is maintained beyond the

translation process. Consequently, an interplay of OXA1L with assembly factors, newly synthesized mitochondrial-encoded proteins, and imported OXPHOS subunits are crucial for proper OXPHOS biogenesis [15,49–54].

For translation, mitochondrial mRNAs engage with the ribosome. It is still unknown how the start codon is recognized on the 5′-UTR-less mRNAs [55]. However, recent structural data on the translation initiation complex indicate that the N-terminal domain of the ribosomal protein mL45 (mL45-NTD) is inserted into the peptide exit tunnel [47,56]. Upon translation switch from initiation to elongation, this domain is displaced. This conformational change during elongation and the movement of the nascent peptide chain through the exit tunnel enables mL45-NTD binding to OXA1L and ribosome engagement with the membrane [47]. The vestibular area in the ribosomal exit tunnel constricts the path and decreases the tunnel width to a minimum in *Paraleucilla magna*, humans, and *Tetrahymena thermophila* mitochondrial ribosome [57]. Therefore, protein folding likely occurs after exit of the polypeptide and could involve OXA. Once the actively-translating ribosome is docked, OXA1L facilitates the translocation of the newly synthesized protein across the inner membrane. In the case of YidC, structural data reveal that the transmembrane domains form a hydrophilic cleft and provide the environment for the translocation of hydrophilic domains across the lipid phase [58,59]. Considering the homology between YidC and OXA1L, it is expected that OXA1L displays a similar arrangement in the membrane. This leads to the question of how the cleft is shielded from the lipids in the membrane. In bacteria, YidC cooperates with the SecYEG translocon and the SecY lateral gate that position close to the hydrophilic groove in YidC during protein translocation [60]. Interestingly, the insertion of single-spanning membrane proteins might be mediated by the hydrophilic groove of monomeric YidC, independent of Sec translocase [59]. However, the issue of how OXA accompanies polypeptides during translocation is unaddressed. Oxa1 has been suggested to homodimerize so that the groove of the second Oxa1 could generate a hydrophilic channel [61,62]. Alternatively, accessory proteins that engage with Oxa1/OXA1L and the nascent chain might stabilize nascent chains during translocation. In agreement with the latter view, although Oxa1/OXA1L is described as the general insertase for newly synthesized proteins, there is increasing evidence that its function and specificity are regulated by associated factors (Figure 2). Such proteins can be specific for a particular translation product or be shared between different early OXPHOS assembly intermediates [50,52,53,63]. Such early assembly factors that stabilize the nascent chain could associate with OXA1L prior to the recognition and binding by the translating ribosome, as in the case of the COX1-dedicated C12ORF62 [48]. Therefore, OXA1L might exist as part of different scaffolds specialized during and after the insertion of a complex core component of OXPHOS; a hypothesis that requires further investigation (Figure 2).

Protein quality control linked to OXA-mediated transport

The functionality of protein import machineries and that of the imported proteins are safeguarded by protein quality control (PQC) systems. One example is ER-associated degradation [64]. In mitochondria, PQC at the outer mitochondrial membrane is currently the best-analyzed pathway [65]. The import through the translocase of the outer membrane (TOM) is controlled by the mitochondrial protein translocation-associated degradation (mitoTAD) pathway [66]. In addition, Msp1/ATAD1 can remove precursors stalled at the TOM and mistargeted tail-anchored proteins inserted in the outer mitochondrial membrane [67–69]. Notably, no such mechanism has been described at the inner mitochondrial membrane translocases. The coisolation of the AAA-protease YME1L with OXA1L has been reported [15]. In the absence of OXA1L proteins that are not inserted into the lipid phase are prone to degradation by the AFG3L2 protease in the inner membrane [70]. These findings suggest that the AAA proteases at the inner membrane might be involved in OXA1-associated quality control, but the underlying mechanism remains unaddressed.

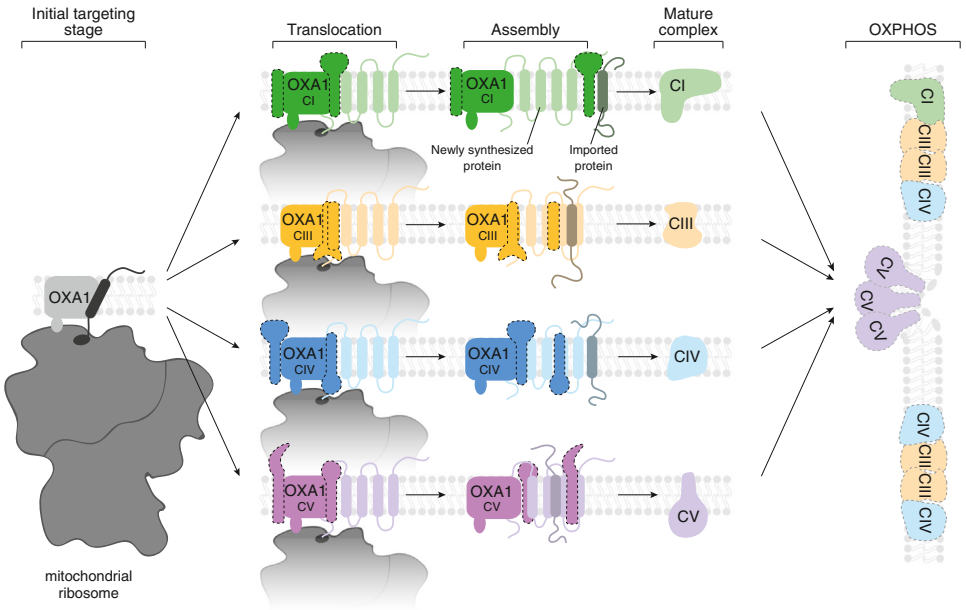

Figure 2. Schematic representation of oxidase assembly (OXA)1 acting as a versatile module for translation, insertion, and assembly of oxidative phosphorylation (OXPHOS) proteins. The translating mitochondrial ribosome associates with OXA1 at the inner mitochondrial membrane in the initial targeting stage. Depending on the translated mitochondrial-encoded protein of a given OXPHOS complex, different accessory factors interact with OXA1 during cotranslational protein insertion to form specific translocation complexes. OXA1 likely remains associated with the newly synthesized protein. Together with other factors, OXA1 might facilitate the assembly of mitochondrial-encoded proteins with nuclear-encoded subunits after membrane insertion. With the subsequent incorporation of the remaining set of subunits, this process yields the mature complex. Subsequently, the intermediated complexes are combined to generate functional OXPHOS complexes and supercomplexes.

Outstanding questions

What is the mechanism of OXA-mediated protein membrane insertion?

How many different OXA-containing complexes exist in the inner mitochondrial membrane that facilitate specific transport of mtDNA-encoded subunits? Which other factors are involved?

Does a quality control system for OXA-mediated import exist, and if so, what are its constituents and mechanism?

Concluding remarks

The Oxa1/OXA1L insertase represents a central protein transport module in the inner mitochondrial membrane functionally adapting to the handled cargos in different interaction networks. It can handle polypeptides synthesized in the cytosol or the mitochondrial matrix. Oxa1/OXA1L engages with mitochondrial ribosomes for cotranslational protein insertion into the inner mitochondrial membrane. In addition, Oxa1/OXA1L recruits membrane proteins that stabilize nascent chains during membrane insertion. With these liaising factors linking Oxa1/OXA1L to an assembly pipeline for a specific translation product and OXPHOS complex, Oxa1/OXA1L supports the biogenesis process. In addition, Oxa1/OXA1L also facilitates the insertion of transmembrane spans post-translationally from the matrix side to obtain the correct membrane organization (see Outstanding questions).

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Declaration of interests

The authors declare no competing interests.

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