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Oxa1 Directly Interacts with Atp9 and Mediates Its Assembly into the Mitochondrial F₁Fₒ-ATP Synthase Complex

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The yeast Oxa1 protein is involved in the biogenesis of the mitochondrial oxidative phosphorylation (OXPHOS) machinery. The involvement of Oxa1 in the assembly of the cytochrome oxidase (COX) complex, where it facilitates the cotranslational membrane insertion of mitochondrially encoded COX subunits, is well documented. In this study we have addressed the role of Oxa1, and its sequence-related protein Cox18/Oxa2, in the biogenesis of the F₁Fₒ-ATP synthase complex. We demonstrate that Oxa1, but not Cox18/Oxa2, directly supports the assembly of the membrane embedded Fₒ-sector of the ATP synthase. Oxa1 was found to physically interact with newly synthesized mitochondrially encoded Atp9 protein in a posttranslational manner and in a manner that is not dependent on the C-terminal, matrix-localized region of Oxa1. The stable manner of the Atp9/Oxa1 interaction is in contrast to the cotranslational and transient interaction previously observed for the mitochondrially encoded COX subunits with Oxa1. In the absence of Oxa1, Atp9 was observed to assemble into an oligomeric complex containing F₁-subunits, but its further assembly with subunit 6 (Atp6) of the Fₒ-sector was perturbed. We propose that by directly interacting with newly synthesized Atp9 in a posttranslational manner, Oxa1 is required to maintain the assembly competence of the Atp9⁻F₁-subcomplex for its association with Atp6.

INTRODUCTION

Oxa1 is a protein of the mitochondrial inner membrane that is involved in the biogenesis of complexes of the oxidative phosphorylation machinery (OXPHOS). Oxa1 is best characterized for its role in the biogenesis of the cytochrome oxidase complex (COX), as it was initially identified in the yeast Saccharomyces cerevisiae as an assembly factor of this complex (Oxidase assembly I; Bauer et al., 1994; Bonnefoy et al., 1994). COX is the terminal electron acceptor complex and thus a key component of the mitochondrial OXPHOS machinery. Analysis of oxa1 null mutant (Δoxa1) mitochondria indicated a complete loss of detectable COX complexes, and hence oxa1 mutants display a respiratory deficient phenotype (Bauer et al., 1994; Bonnefoy et al., 1994). Further analysis of a temperature-sensitive oxa1 mutant, the pet ts1402 strain, indicated that the COX complex assembly defect observed in the absence of Oxa1 function is attributed to a failure of the mitochondrially encoded COX subunits, subunits 1 and 2 (Cox1 and Cox2, respectively), key subunits of the COX complex, to become inserted into the membrane in the absence of the Oxa1 protein (Hell et al., 1997, 1998, 2001). A direct role for Oxa1 in facilitating the membrane insertion process of these proteins was further indicated by the demonstration that Oxa1 physically interacts in a cotranslational manner with the mitochondrially encoded COX subunits, i.e., as they were being synthesized on ribosomes within the mitochondrial matrix and undergoing membrane insertion (Hell et al., 1998, 2001). In addition evidence for a direct interaction of Oxa1 with the mitochondrial ribosomes, which is supported through the matrix-localized C-terminal region of Oxa1, has also been presented (Jia et al., 2003; Szyrach et al., 2003).

Closer examination of oxa1 null mutant mitochondria has demonstrated that the function of Oxa1 is not limited to the assembly of the COX complex, but that it is also involved in the biogenesis of other OXPHOS complexes. Published reports indicate the enzyme activities of the cytochrome bccoli and F₁Fₒ-ATP synthase complexes are significantly reduced in the Δoxa1 mitochondria (Altamura et al., 1996; Szyrach et al., 2003). However, in contrast to the COX complex, which is completely absent in Δoxa1 mitochondria, the cytochrome bccoli complex and F₁Fₒ-ATP synthase complex activities, although strongly reduced, are clearly still detectable in Δoxa1 mitochondria (Altamura et al., 1996). Consistently, the insertion of mitochondrially encoded subunits of these complexes, cytochrome b and Atp6, respectively, was found to be only partially impaired in mitochondria isolated from the pet ts1402 oxa1 strain, in contrast to Cox1 and Cox2 subunits, where the insertion process was almost completely blocked (Hell et al., 2001). Thus the presence of measurable levels of cytochrome bccoli and ATP synthase enzymes in Δoxa1 mitochondria suggests that, in contrast to the COX complex, Oxa1 does not play an essential role in the assembly of these OXPHOS complexes. It is presently unknown whether the translocase function of Oxa1 is required for the biogenesis of the ATP synthase complex or whether Oxa1 may function in some other capacity to assist in the assembly of this complex.

Oxa1 is a member of the conserved Oxa1/YidC/Alb3 protein family, which is found throughout prokaryotes and eukaryotes (Yen et al., 2001; Stuart, 2002; Kuhn et al., 2003). The bacterial members (YidC) of this family have been shown to mediate the insertion of a number of membrane...
proteins including subunits of the cytochrome oxidase (CyoA) and subunit c (equivalent to mitochondrial Atp9) of the F<sub>i</sub>F<sub>o</sub>-ATP synthase complexes (van Bloois et al., 2004, 2006; van der Laan et al., 2004, 2005; Celebi et al., 2006; Kol et al., 2006; du Plessis et al., 2006). The Alb3 protein members are localized in the thylakoid membrane system of chloroplasts and the role of Alb3 in the insertion of a number of photosynthetic membrane proteins has been documented (Sundberg et al., 1997; Moore et al., 2000; Gerdes et al., 2006). Thus the Oxa1/YidC/Alb3 family represents an evolutionarily conserved group of proteins involved in facilitating critical steps to ensure the integration of proteins into diverse biological membrane systems (Kuhn et al., 2003). Recently the Oxa2/Cox18 protein was proposed to form a new subfamily of the Oxa1/YidC/Alb3 proteins. Members of this group, represented by Cox18 in the yeast S. cerevisiae, and Oxa2 in Neospora crassa, have been shown to play a critical role in the biogenesis of the COX complex (Souza et al., 2000; Saracco and Fox, 2002; Funes et al., 2004; Preuss et al., 2005). The Cox18 protein is required for the assembly of the COX complex, as it has been reported to be involved in facilitating the export of the C-terminal region of the Cox2 protein from the matrix across the inner membrane to the intermembrane space (Saracco and Fox, 2002). Thus the role of Cox18/Oxa2 proteins in protein translocation has been proposed to overlap with that of its sequence-related Oxa1. Oxa1 however has been proposed to play a pivotal role in the cotranslational insertion of the mitochondrially encoded COX subunits, whereas the Cox18/Oxa2 protein may be functionally different, possibly by being limited to performing posttranslational insertion events (Preuss et al., 2005). In this respect, it is noteworthy that the matrix-localized C-terminal region of Oxa1 which supports the ribosome association is not conserved among the Cox18/Oxa2 protein family members.

The precise function of Oxa1 and its sequence-related Cox18/Oxa2 protein, in the assembly of the ATP synthase is currently unknown and is the central theme of this study. The F<sub>i</sub>F<sub>o</sub>-ATP synthase complex can be divided into two functionally distinct parts, the hydrophilic F<sub>i</sub>-part, which performs the ATP synthesis and hydrolysis events, and the membrane-embedded F<sub>o</sub>-sector, which facilitates the passage of protons across the inner membrane. The F<sub>i</sub>-sector is composed entirely of nuclear-encoded proteins, whereas the F<sub>o</sub>-complex is assembled from both nuclear and mitochondrially encoded proteins. The mitochondrially encoded Atp6, Atp8, and Atp9 are all subunits of the F<sub>o</sub>-sector. The Atp9 subunit spans the inner membrane twice in an N<sub>out</sub>-C<sub>out</sub> orientation and assembles to form a homo-oligomeric ring structure composed of 9–12 subunits. The Atp9 oligomer directly interacts and cooperates with Atp6 to mediate the transport of protons across the membrane, the driving force for ATP synthesis by the coupled F<sub>i</sub>-sector (Stock et al., 1999; Devenish et al., 2000; Velours and Arselin, 2000; Capaldi and Agerler, 2002). During the assembly of the ATP synthase complex, the newly synthesized Atp9 has been shown to oligomerize and form an F<sub>i</sub>-containing subcomplex before associating with the Atp6 protein (see Ackerman and Tzagoloff, 2005 for recent review). The assembly of Atp6 with the F<sub>i</sub>-Atp9 subcomplex thus represents a late step in the assembly pathway of the F<sub>i</sub>F<sub>o</sub>-complex. The inner membrane protein Atp10 has been shown to act as an Atp6-specific chaperone and by directly interacting with the newly synthesized Atp6 protein Atp10 coordinates the assembly of Atp6 with the F<sub>i</sub>-Atp9 subcomplex (Herrmann et al., 1994; Tzagoloff et al., 2004).

The involvement and possible overlapping functions of Oxa1 and Cox18/Oxa2 in the assembly of the yeast S. cerevisiae ATP synthase therefore were directly analyzed in this study. We present data here demonstrate that Oxa1 plays an important, but nonessential, role in the assembly of the F<sub>i</sub>-sector of the ATP synthase complex. Our data indicate that Cox18/Oxa2, unlike Oxa1, is not required for ATP synthase assembly, however. In addition, our results shed light on how Oxa1 may function to mediate the assembly of the F<sub>i</sub>-sector. We demonstrate that Oxa1 can directly interact with Atp9 after its translation in mitochondria and in a manner that does not require the matrix-exposed C-terminal region of Oxa1. We propose that the interaction of Atp9 with Oxa1 is necessary to ensure the efficient and correct assembly of Atp9 oligomers and in such a manner that they are competent to subsequently assemble together with Atp6 in the membrane. In the absence of Oxa1, Atp9 fails to correctly assemble with Atp6 efficiently, and this has severe consequences for the levels of enzymatically active F<sub>i</sub>F<sub>o</sub>-ATP synthase complexes in oxa1-deficient mutants. Finally, we show here that Oxa1 can be purified with ATP synthase complex, highlighting the affinity that Oxa1 has for this complex.

**MATERIALS AND METHODS**

**Yeast Strains**

Yeast strains used in this study were wild-type W303-1A (Mata, leu2, trp1, ura3, his3, ade2), rho<sup>0</sup> W303-1A (Mata, leu2, trp1, ura3, his3, ade2), and the oxa1-null mutant, Δoxa1 (W303-1A, leu2, trp1, ura3, his3, ade2; OXA1::KAN<sup>+</sup>), where the complete OXA1 open reading frame (ORF) was replaced by the kanamycin resistance gene (KAN<sup>+</sup>) using homologous recombination (Wach et al., 1994). Construction of the strains Δoxa1 and Δoxa1, Δoxa18 was performed by the introduction of the HIS3 gene into the COX18 locus of wild-type and Δoxa1::KAN<sup>+</sup> cells, resulting in the replacement of the entire COX18 ORF. Correct homologous recombination of the HIS3 or KAN<sup>+</sup> gene at their respective gene locus was verified by PCR analysis (results not shown). Construction of the Δap1 strain was performed by introducing the LEU2 auxotrophic gene into the wild-type cells, resulting in the replacement of the ORF of the IMP1 ORF (Cruciat et al., 2000). Strains expressing full-length Oxa1 and Oxa1<sub>His</sub> from the galactose-inducible GAL10 promoter have been previously described (Jia et al., 2003). Construction of the strain expressing histidine-tagged C-terminally truncated Oxa1 (Oxa1Δ338) was achieved as follows. A DNA fragment encoding for a C-terminally truncated Oxa1 (codons 1-318) followed by 12 histidine-encoding codons and a translational stop codon was generated by PCR. This fragment was then cloned into a yeast-integrating vector Yip351-GAL10 (LEU2) and downstream of the galactose-inducible promoter GAL10. The resulting plasmid Yip351-GAL10::OXA1ΔHis was cloned into the yeast genome of the oxa1-null mutant at the LEU2 locus after linearization with BstEII.

**H<sub>2</sub>O<sub>2</sub> Treatment and Dispersal of Atp9 Oligomer to Monomers**

Mitochondria (200 μg) in 20 mM Tris-HCl, pH 8.0, buffer were mixed with H<sub>2</sub>O<sub>2</sub>, final concentration 4% (vol/vol). The samples were shaken at 25°C for 10 min. SDS sample buffer (Laemmli, 1970) was added, and samples were heated at 95°C for 5 min before loading the gel. This treatment with H<sub>2</sub>O<sub>2</sub> resulted in an efficient dispersal of the SDS-resistant Atp9 oligomers into monomeric subunits (results not shown).

**Digitonin Solubilization of Mitochondria and Clear Native Gel Electrophoresis**

Clear native-PAGE (CN-PAGE) analysis of F<sub>i</sub>F<sub>o</sub>-ATP synthase complexes after solubilization with digitonin was performed essentially as described previously (Wittig and Schägger, 2005). Mitochondria (200 μg of protein) were lysed with 36 μl of digitonin buffer (34 mM potassium acetate, 34 mM HEPES-KOH, pH 7.4, 11.4% glycerol, 1 mM phenylmethylsulfonyl fluoride) and digitonin (at concentrations indicated) for 30 min on ice and subjected to a clarifying spin (JA-25.50 rotor, Beckman Avanti J-25 system (Fullerton, CA); 30 min, 30,000 × g). The supernatants were collected, and 2 μl of 50% glycerol was added to the samples before electrophoresis. Samples were analyzed by CN-PAGE using a 3.5–10% gradient gel.

**In Gel Oligomycin-sensitive ATPase Activity Assay**

F<sub>i</sub>F<sub>o</sub>-ATPase activity assay was performed after CN-PAGE, as previously described (Wittig and Schägger, 2005). Native gels were incubated for 3 h in...
a buffer containing 270 mM glycine, 35 mM Tris-HCl, pH 8.4. In a parallel assay, additional oligomycin (5 \mu g/ml) was supplemented in the buffer. The ATP hydrolysis assay was carried out in 270 mM glycine, 35 mM Tris-HCl, 8 mM ATP, 14 mM MgSO4, 0.2% Pb(NO3)2, pH 8.4, and stopped after 5-10 min by a 30 min incubation in 50% methanol, 50% water, followed by a 30 min incubation in water.

In Organello Labeling of Mitochondrial Translation Products

In organello labeling of mitochondrial translation products was performed in isolated mitochondria for 30 min at 25°C, essentially as described previously (Hell et al., 2001). After translation and chase in the presence of cold methionine and puromycin for 30 min, mitochondria were lysed in SDS-sample buffer (Laemmli, 1970). Translation products were analyzed by SDS-PAGE and autoradiography.

Ni-NTA Purification of Oxa1His Complex

Mitochondria harboring the GAL10 expressed Oxa1His derivative (200 \mu g) were solubilized in 200 \mu l of TNT buffer (1% Triton X-100, 300 mM NaCl, 60 mM Tris-HCl, pH 7.4) for 5 min on ice. After a clarifying spin (20,800 \times g, 15 min at 4°C), the supernatants were incubated for 1 h at 4°C with the Ni-NTA beads (equilibrated in the TNT buffer containing 30 mM imidazole). The beads were washed with TNT-imidazole buffer, and purified proteins were eluted with SDS sample buffer containing 5 \%(vol/vol) \beta-mercaptoethanol and 0.5 M imidazole followed by SDS-PAGE analysis. When indicated, mitochondria were initially solubilized with 0.1% SDS, and a buffer containing 0.1% SDS instead of Triton X-100 was used.

Gel Filtration Analysis

Mitochondria (200 \mu g protein) were solubilized in 200 \mu l of buffer containing 1% Triton X-100, 300 mM NaCl, and 60 mM Tris-HCl, pH 7.4, for 5 min on ice. After a clarifying spin (100,000 \times g, 15 min at 4°C), the supernatant was loaded to the Superose 12 column. Samples were run with buffer containing 0.1% Triton X-100, 300 mM NaCl, and 60 mM Tris-HCl, pH 7.4, at the speed of 0.1 ml/min, and 0.5-ml/fraction eluent was collected. Proteins were trichloroacetic acid (TCA) precipitated from each fraction and analyzed by SDS-PAGE. Immunoblotting was performed to analyze the presence of Atp9, Atp6, and Atp8.

Miscellaneous

Mitochondria were isolated from cultures grown at 30°C in YP-0.5% lactate, 2% galactose medium. Isolation of mitochondria was performed as described previously (Jia et al., 2003).

RESULTS

The Assembly of the F1F0-ATP Synthase Is Perturbed in the Absence of Oxa1, But Not Cox18

To investigate the requirement for Oxa1 and Cox18 in the biogenesis of the ATP synthase enzyme, we analyzed both the enzymatic activity and assembly state of ATP synthase complexes in mitochondria isolated from S. cerevisiae yeast mutants deficient in Oxa1 and/or Cox18 (Figure 1A). Mitochondria isolated from the single deletion strains \(\Delta oxa1\) and \(\Delta cox18\) and also from the double gene knockout strain \(\Delta oxa1\), \(\Delta cox18\) were solubilized with the detergent digitonin, and the resulting extracts were subjected to native PAGE (CN-PAGE) analysis. The oligomycin-sensitive enzymatic activity of the ATP synthase complexes was assayed after CN-PAGE and using an in-gel ATP hydrolysis assay, in the presence or absence of the F1F0-ATP synthase specific inhibitor oligomycin (Figure 1A). In parallel, mitochondria isolated from the isogenic wild-type strain and an independent cytochrome oxidase–deficient strain, the \(\Delta imp1\) strain, were also analyzed, as controls.

Under the digitonin lysis conditions used, the active and oligomycin-sensitive F1F0-ATP synthase enzyme complex from wild-type mitochondria was solubilized and resolved on CN-PAGE as a combination of dimeric and monomeric complexes, consistent with previously published observations (Arnold et al., 1998; Saddar and Stuart, 2005). The observed monomeric F1F0-ATP synthase complexes are thought to be a result of a dissociation of some ATP synthase

![Figure 1](image-url) Characterization of assembly defects of ATP synthase in \(\Delta oxa1\) and \(\Delta cox18\) mutants. (A) Mitochondria from wild-type (WT), \(\Delta oxa1\), \(\Delta cox18\), \(\Delta oxa1\), \(\Delta cox18\), and \(\Delta imp1\) strains were solubilized with buffer containing 2% digitonin and subjected to CN-PAGE followed by in-gel ATPase activity assay in the absence or presence of oligomycin (5 \mu g/ml, + or − oligomycin, as indicated). The position of the dimeric \(V_{dim}\) and monomeric \(V_{mon}\) forms of the F1F0-ATP synthase are indicated. (B) Mitochondria from WT and \(\Delta oxa1\) strains were solubilized with either 1 or 2% digitonin as indicated subjected to CN-PAGE, and Western blotted. ATP synthase complexes were detected with an anti-F1 antiserum. The presence of F1-containing subcomplexes in the \(\Delta oxa1\) mitochondria are indicated by an asterisk (*). (C) Mitochondria from rho0, WT, \(\Delta oxa1\), \(\Delta cox18\), \(\Delta imp1\), and \(\Delta cox18\), \(\Delta imp1\) strains were subjected to SDS-PAGE and Western blotted using antisera against F1 subunit Atp6, F1 subunits Atp4, Atp6, Atp9, and the inner membrane protein Tim23. For the analysis of the Atp9 levels, mitochondria were treated with H2O2 (see Materials and Methods) to disperse SDS-resistant Atp9 oligomers to monomeric proteins, before the SDS-PAGE analysis.
specifically on the Fo-sector, where it is thought to bind to complexes in the /H9004/H9004 were oligomycin sensitive, were clearly detected in the correct and functional assembly of the coupled F1Fo-complex. This inhibition is an indicator of the molecular weight F1-complexes, corresponding to partially bled F1-complexes was also observed in the wild-type control mitochondria. Thus it would appear that the reduction in both the protein and enzyme activity levels of the assembled F1Fo-ATP synthase complex in the absence of Oxa1, but not in the absence of Cox18. We conclude therefore that Oxa1, unlike Cox18, plays an important role in the biogenesis of the enzymatically active F1Fo-ATP synthase complex.

F0-Sector Subunits Atp4 and Atp6, But Not Atp9, Are Strongly Reduced in the Absence of Oxa1

Given that Oxa1 has been described to be involved in facilitating the insertion of proteins into the inner membrane, we analyzed directly the steady state levels of ATP synthase subunits of the membrane embedded F0-sector in the /H9004/H9004 mitochondria. Mitochondria isolated from the single null mutant strains, /H9004/Oxa1 and /H9004/Cox18 and also from the double knockout strain /H9004/Oxa1, /H9004/Cox18 were analyzed by SDS-PAGE and Western blotting. Wild-type, COX-deficient Deltaimp1 and rho0 (contain no mtDNA) mitochondria were also analyzed in parallel as controls. Immunodecorated was subsequently performed to assess the steady state levels of the F0-sector subunits Atp4, Atp6, and Atp9 and also the F0-sector subunit, Atpβ (Figure 1C). The level of Tim23, an inner membrane protein and component of the TIM23 translocase, was analyzed as a loading control for the mitochondria.

Consistent with the CN-PAGE results, the levels of Atp4, Atp6, and Atp9 were not adversely affected in the /H9004/Oxa1 mitochondria, because they were similar to those observed in the wild-type-Deltaimp1 mitochondria. In the /H9004/Oxa1 mitochondria, however, the steady state levels of Atp4 and Atp6 were significantly reduced. Although reduced relative to wild type, the levels of Atp9 in the mitochondria isolated from the /H9004/Oxa1 strain were not so dramatically affected as those of Atp6 or Atp4 proteins. The levels of Atp4, Atp6, and Atp9 in the /H9004/Oxa1, /H9004/Cox18 double mutant were similar to those of the /H9004/Oxa1 single mutant, again supporting the conclusion that Cox18 does not contribute to the assembly or stability of the ATP synthase in the absence of Oxa1. Note we were unable to assess the steady state levels of the other mitochondrially encoded F0-sector subunit, Atp8, because an antibody specific for the Atp8 protein was not available. In contrast to the F0-sector subunits, the F0-sector subunit, Atpβ was not so significantly reduced in the /H9004/Oxa1 mitochondria. Thus it would appear that the reduction in assembled F1Fo-ATP synthase complexes in the absence of Oxa1 reflects the limiting levels of F0-sector, rather than F1-sector subunits.

Oxa1 Associates Specifically with Newly Synthesized Atp9

We investigated next whether Oxa1 directly plays a role in the assembly of the ATP synthase complex by physically interacting with mitochondrially encoded subunits of the F0-sector of the ATP synthase complex in a posttranslational manner and before their assembly into the F1Fo-ATP synthase complex. Three of the F0-sector subunits are encoded by the mitochondrial DNA: Atp6, Atp8, and Atp9. To address a possible association between Oxa1 and these subunits of the F0-sector, we performed an in organello translation in the presence of [35S]methionine using mitochondria isolated from the wild-type strain or from strains expressing authentic Oxa1 or the Oxa1His-tagged derivatives (Figure 2A). The pattern of protein translation products was similar between the different mitochondrial types with the exception of a number of higher molecular mass radiolabeled bands (range 55–75 kDa), which correspond to SDS-resistant double mutant, but not, however, in the /H9004/Oxa1 single null mutant mitochondria (results not shown).
forms of the mitochondrialy synthesized proteins. Two prominent bands (70 and 72 kDa) were observed in the mitochondria harboring the overexpressed Oxa1 and Oxa1_{His} proteins, respectively. A weaker 70-kDa band was observed in the wild-type mitochondria. The shift in the electrophoretic mobility of this band in the mitochondria harboring the His-tagged derivative of Oxa1 suggested that these additional radiolabeled bands represent an SDS-resistant complex between a mitochondrialy encoded protein and Oxa1. The mobility difference between the two forms of this band (between the Oxa1 and Oxa1_{His} mitochondria) are accounted for by the presence of the additional 12 histidine residues (1.7 kDa in mass) of the Oxa1_{His} derivative.

To further verify that this radiolabeled band represents an Oxa1-containg complex and to ascertain the identity of the radiolabeled associating protein, we took advantage of its SDS-stability and the formation of the complex in mitochondria harboring the His-tagged version of Oxa1. In organello translation was performed in mitochondria bearing the Oxa1_{His} protein, and after translation mitochondria were lysed in an SDS-containing buffer, and the solubilized proteins were subjected to Ni-NTA chromatography. Mitochondria containing overexpressed wild-type Oxa1, i.e., nontagged Oxa1, were analyzed in parallel as a control. The Ni-NTA purified proteins were analyzed either directly by SDS-PAGE or after a TCA-precipitation step. The TCA precipitation step has been previously described to disperse SDS-resistant complexes of mitochondrial translation products (Herrmann et al., 1994; Tzagoloff et al., 2004). The SDS-resistant radiolabeled complex was recovered on the Ni-NTA beads, when translation was performed in the mitochondria bearing the His-tagged Oxa1 derivative, but not when translation was performed in the control mitochondria bearing the expressed nontagged version of Oxa1 (Figure 2B). This result confirms that the radiolabeled complex was indeed an Oxa1_{His}-containing complex. TCA precipitation of this purified material resulted in the release of one radiolabeled species, which from its electrophoretic behavior was determined to be Atp9, using a gel system that resolves the Atp9 protein from the closely migrating Atp8 protein. In addition, using this gel system, the affinity-purified radiolabeled Atp9 protein was shown to comigrate with endogenous Atp9 protein, which was recognized by Atp9-specific antibodies (results not shown, and also see Figure 6). No evidence to suggest that this complex contained Atp6 (or the other mitochondrially encoded proteins), in addition to Atp9, was obtained. Thus we conclude that after translation in mitochondria, newly synthesized Atp9 remains in association with Oxa1, where it can form an SDS-resistant complex.

**Assembly of Atp9 Oligomer Is Affected in Absence of Oxa1**

Given that Oxa1 associates with newly synthesized Atp9 in a posttranslational manner, we next addressed if Oxa1 is involved in the assembly of Atp9 into the F1F0-ATP synthase complex. During its assembly pathway, Atp9 forms a homooligomeric complex, which then associates with F1-sector subunits (Herrmann et al., 1994; Tzagoloff et al., 2004). We term this F1-containing Atp9-assembly intermediate here the Atp9-F1 subcomplex. Formation of the Atp9-F1 subcomplex has been shown to precede the Atp10-dependent step of coassembly of Atp9 with Atp6 (Herrmann et al., 1994; Tzagoloff et al., 2004). As reported earlier, the steady state levels of Atp9, in contrast to other F1-sector subunits, appear not to be drastically reduced in the absence of Oxa1. We hypothesized therefore that Atp9 can assemble to an intermediate

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**Figure 2.** Oxa1 associates specifically with newly synthesized Atp9. (A) In organello translation was performed in the presence of \[^{35}S\]methionine for 30 min at 25°C in mitochondria isolated from the wild-type (WT) yeast strain, a strain overexpressing Oxa1 (GAL-Oxa1) and from a strain expressing histidine-tagged Oxa1 (GAL-Oxa1_{His}). Incorporation of \[^{35}S\]methionine was stopped by addition of excess unlabeled methionine, and the mitochondria were solubilized in SDS buffer and subjected to SDS-PAGE. The bands marked with an asterisk (*) indicated the complex formed by Oxa1 and a translation product. The positions of the molecular mass standards and mitochondria-encoded proteins, Var1, Cox1, Cox2, cytochrome \(b\) (Cytb), Cox3, Atp6, Atp8, and Atp9, are indicated. (B) In organello translation was performed in mitochondrial harboring overexpressed Oxa1 or Oxa1_{His}, as described in A. The complex formed between Oxa1_{His} and the radiolabeled translation products were purified by Ni-NTA chromatography and either loaded directly on the gel (−TCA) or after TCA precipitation (+TCA). The mobility of the SDS-resistant Oxa1_{His}-Atp9 complex and the monomeric Atp9 protein after release by TCA precipitation are indicated.
stage and be at least partially stabilized in the absence of Oxal1.

We therefore addressed the assembly state of the endogenous Atp9 in the Oxal1 mitochondria and compared it to that of Atp9 accumulated in the Atpt10 mitochondria (Figure 3). Wild-type and rho0 mitochondria were also analyzed in parallel as controls. Mitochondria were lysed using Triton X-100, a detergent that maintains the stability of the monomeric form (but not the dimeric form) of the F1F0-ATP synthase, and the assembly state of the Atp9 subunit was analyzed by size exclusion chromatography (Figure 3). In wild-type mitochondria, the majority of Atp9 was present in the monomeric ATP synthase complex, because it coeluted from the column together with Atp6 and Atpβ, in fractions 15–18, corresponding to a complex of ~550 kDa. Some Atp9 eluted in later fractions together with a low level of Atpβ (fractions 19 and 20), with a molecular mass of ~300 kDa, and most likely corresponds to the previously described Atp9-F1 subcomplex. Atp6 was found exclusively with the fully assembled ATP synthase complex, however (Figure 3A).

In the Oxal1 mitochondria, the majority of Atp9 eluted from the column with some of the Atpβ protein, in fractions 19 and 20, which correspond to the ~300-kDa Atp9-F1 subcomplex. Only a minor fraction of Atp9 eluted from the column in the fractions (15–18) corresponding to fully assembled F1F0-ATP synthase, as indicated by the coelution of Atp6 and Atpβ (Figure 3B). The observed low level of fully assembled F1F0-ATP synthase complexes in the Oxal1 strain is consistent with the previous CN-PAGE results (Figure 1). Hence only a small fraction of the total Atpβ subunit was present in the fractions corresponding to the fully assembled F1F0-ATP synthase complex; the majority of Atpβ eluted from the column either with Atp9-F1 subcomplex (fractions 19 and 20) or later in F1-complex of ~150 kDa (fractions 21–23). The latter F1-complex was similar in mass as an Atpβ-containing assembly intermediate that could be observed in the rho0 mitochondria, which lack all of the mtDNA-encoded F1-sector subunits, Atp9, Atp8, and Atp6 (Figure 3D). We conclude from these results that in the Oxal1 mitochondria, the Atp9 protein, which does not assemble into the complete F1F0-ATP synthase complex, accumulates in an Atp9-F1 subcomplex. The behavior of the Atp9 in the Oxal1 mitochondria was similar to that in the Atpt10 mutant mitochondria, where the Atp9 protein also accumulated in a 300-kDa complex (Figure 3C). As mentioned earlier, assembly of Atp9 into its homo-oligomer, followed by its association with F1-subunits, occurs in an Atpt10-independent manner; but further assembly of this oligomer with Atp6 does not occur in the absence of Atpt10, thus preventing the complete assembly of the F1F0-ATP synthase complex. The similarity of the behavior of Atp9 in Oxal1 and the Atpt10 mitochondria, i.e., competence to assemble in a stable

![Figure 3](image-url)

**Figure 3.** Atp9 assembles into an F1-containing subcomplex in the absence of Oxal1. Mitochondria from wild-type (WT; A), Oxal1 (B), Atpt10 (C), and rho0 (D) strains were solubilized with 1% Triton X-100 containing buffer and subjected to size exclusion chromatography on Superose 12 column. Fractions (11–27) eluted from the column were TCA precipitated and subjected to SDS-PAGE followed by Western blotting with antisera against F1 subunit Atpβ and F0 subunits Atp6 and Atp9. The elution profile of the molecular mass standards 443, 200, 150, 66, and 29 kDa are indicated in the wild-type panel. Note the exposure time for Atp6 in the Oxal1 and Atpt10 mitochondria was significantly longer than that for the wild-type control, because of the strongly decreased levels of Atp6 in these mutant mitochondria.

![Figure 4](image-url)

**Figure 4.** Assembly of the ATP synthase complex is impaired in Oxal1 mitochondria. Mitochondrial from wild type (A and B) and Oxal1 (B) strains were subjected to translation and radiolabeled for 30 min at 25°C followed by a chase period in the presence of excess cold methionine, an ATP-regenerating system and in the absence or presence of oligomycin, as indicated. Mitochondria were reisolated, lysed and subjected to SDS-PAGE. (A) Western blotting, and autoradiography. The positions of the 54-kDa SDS-resistant complex between Atp9 and Atpt6, and the 48-kDa Atp9-oligomer, are indicated, by 54k and 48k, respectively. (B) The levels of both the 54-kDa (dark gray bars) and 48-kDa (light gray bars) complexes were quantified by Phosphorimaging and are each expressed as a percentage of the total, i.e., the sum of the 54- and 48-kDa signals.
ATP synthase subunits Atp analyzed by SDS-PAGE and Western blotting with antisera against incubated with Ni-NTA beads. The Ni-NTA–purified material was lysed with buffer containing 1% Triton X-100, as indicated, and were

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A. (A) Mitochondria isolated from strains expressing Oxa1His or Oxa1 were solubilized with buffer containing either 1% Triton X-100 (TX-100) or 0.1% SDS as indicated and incubated with Ni-NTA beads as described in A. (B) Mitochondria isolated from strains expressing Oxa1His or Oxa1 were solubilized with buffer containing either 1% Triton X-100 or 0.1% SDS as indicated and incubated with Ni-NTA beads. The Ni-NTA–purified material was analyzed by SDS-PAGE and silver-staining. (B) Mitochondria isolated from strains expressing Oxa1His or Oxa1 (GAL-Oxa1 and GAL-Oxa1His, respectively) were solubilized with buffer containing 1% Triton X-100 in the absence or presence of oligomycin (10 μg/ml) as indicated. Samples were further analyzed by analyzing its ability to form SDS-resistant oligomers in Δoxa1 mitochondria after its synthesis (Figure 4). When [35S]methionine-labeling of mitochondrial translation products is performed in isolated wild-type mitochondria, in addition to the eight mitochondrially encoded proteins, two additional slower migrating radiolabeled bands, with electrophoretic mobilities corresponding to the masses of 48- and 54-kDa products, are observed (Figure 4A), as has been previously reported (Herrmann et al., 1994; Tzagoloff et al., 2004). These 48- and 54-kDa products correspond to oligomers of F1-subunits that are released from fully or partially assembled F1F0-ATP synthase complexes, but fail to be completely depolymerized by the SDS, due to the very hydrophobic nature of the ATP9-ATP9 interactions in the F0-oligomer. These SDS-resistant ATP9 complexes can be dispersed into individual monomeric polypeptides through TCA precipitation (Herrmann et al., 1994; Tzagoloff et al., 2004), where it has been established that the 48-kDa product is composed of ATP9 proteins, whereas the 54-kDa product is generated from a later stage assembly intermediate as it contains ATP6, in addition to ATP9 (Tzagoloff et al., 2004). In wild-type mitochondria the level of the 54-kDa oligomer was observed to be greater than that of the 48-kDa complex, indicating efficient assembly of ATP9 with the ATP6 protein in the presence of Oxa1 (Figure 4B). The level of the 54-kDa complex observed, but of not the 48-kDa oligomer, was partially affected in the presence of oligomycin, indicating the binding of the ATP synthase–specific inhibitor may adversely affect the formation of the ATP9-ATP6 complex (Figure 4B). Assembly of the 48-kDa ATP9-containing complex was observed in the Δoxa1 mitochondria, indicating the ability of ATP9 to oligomerize in the absence of Oxa1, a result consistent with the previously described gel filtration analysis. In contrast however, to the wild-type control mitochondria, formation of the 54-kDa complex was perturbed in the absence of Oxa1, because a significant decrease in the amount of the 54-kDa product relative to the 48-kDa complex was observed (Figure 4B).

In summary these data support the conclusion that the oligomerization of newly synthesized ATP9 protein may oc-

manner in an ATP9-F1 subcomplex, would suggest that further assembly of the ATP synthase in the absence of Oxa1 is limited by the step of coassembly of the ATP9 oligomer with the ATP6 protein.

Newly Synthesized ATP9 Can Oligomerize in the Absence of Oxa1, But Assembly with ATP6 Is Perturbed

In a second independent approach we tested the ability of newly synthesized ATP9 to assemble in the absence of Oxa1, by analyzing its ability to form SDS-resistant oligomers in Δoxa1 mitochondria after its synthesis (Figure 4). When [35S]methionine-labeling of mitochondrial translation products is performed in isolated wild-type mitochondria, in addition to the eight mitochondrially encoded proteins, two additional slower migrating radiolabeled bands, with electrophoretic mobilities corresponding to the masses of 48- and 54-kDa products, are observed (Figure 4A), as has been previously reported (Herrmann et al., 1994; Tzagoloff et al., 2004). These 48- and 54-kDa products correspond to oligomers of F1-subunits that are released from fully or partially assembled F1F0-ATP synthase complexes, but fail to be completely depolymerized by the SDS, due to the very hydrophobic nature of the ATP9-ATP9 interactions in the F0-oligomer. These SDS-resistant ATP9 complexes can be dispersed into individual monomeric polypeptides through TCA precipitation (Herrmann et al., 1994; Tzagoloff et al., 2004), where it has been established that the 48-kDa product is composed of ATP9 proteins, whereas the 54-kDa product is generated from a later stage assembly intermediate as it contains ATP6, in addition to ATP9 (Tzagoloff et al., 2004). In wild-type mitochondria the level of the 54-kDa oligomer was observed to be greater than that of the 48-kDa complex, indicating efficient assembly of ATP9 with the ATP6 protein in the presence of Oxa1 (Figure 4B). The level of the 54-kDa complex observed, but of not the 48-kDa oligomer, was partially affected in the presence of oligomycin, indicating the binding of the ATP synthase–specific inhibitor may adversely affect the formation of the ATP9-ATP6 complex (Figure 4B). Assembly of the 48-kDa ATP9-containing complex was observed in the Δoxa1 mitochondria, indicating the ability of ATP9 to oligomerize in the absence of Oxa1, a result consistent with the previously described gel filtration analysis. In contrast however, to the wild-type control mitochondria, formation of the 54-kDa complex was perturbed in the absence of Oxa1, because a significant decrease in the amount of the 54-kDa product relative to the 48-kDa complex was observed (Figure 4B).

In summary these data support the conclusion that the oligomerization of newly synthesized ATP9 protein may oc-
cur in the absence of Oxa1, but its further assembly into a F\textsubscript{\textgamma}-sector, a step that involves its interaction with the Atp6 protein, is perturbed in the \Deltaoxa1 mitochondria.

**Oxa1 Copurifies with Components of the F\textsubscript{\textgamma}F\textsubscript{\textalpha}-ATP Synthase**

Given that Oxa1 associates with newly synthesized Atp9 in a posttranslational manner, we investigated whether the Oxa1 complex was also associated with the pre-existing assembled ATP synthase complex in mitochondria (Figure 5). To do so, we used the derivative of Oxa1 bearing a C-terminal histidine tag (Oxa1\textsubscript{His}) expressed in the \Deltaoxa1 strain and performed affinity purification of the Oxa1\textsubscript{His} and its associating proteins, using metal chelating chromatography. Mitochondria harboring the Oxa1\textsubscript{His} derivative were lysed with Triton X-100, and the Oxa1\textsubscript{His} protein was purified on Ni-NTA agarose beads. Oxa1\textsubscript{His} and copurifying proteins were analyzed by SDS-PAGE followed by silver staining (Figure 5A). In addition to Oxa1\textsubscript{His} (38 kDa), a number of other polypeptides were observed to copurify with Oxa1\textsubscript{His}. We verified the identity of these Oxa1-associating proteins as subunits of the ATP synthase complex by either MALDI-TOF analysis (Atp\textsubscript{\alpha} and Atp\textsubscript{\beta}\textsubscript{\alpha} subunits) or through Western blot analysis using subunit specific antibodies (Atp\textsubscript{\alpha}, Atp\textsubscript{\beta}, Atp4, Atp5, Atp6, and Atp9 and subunits f, j, and d; Figure 5B, Su f, Su j and Su d). The recovery of these ATP synthase subunits on the Ni-NTA agarose beads was specific for the presence of the His-tagged version of the protein, because they were not recovered when mitochondria bearing the nontagged version of Oxa1 were used as a control (Figure 5B). Furthermore, the recovery of the ATP synthase subunits on the beads appeared to be due to their specific association with Oxa1\textsubscript{His}, because when mitochondrial lysis and purification was performed using the more stringent detergent SDS only Oxa1\textsubscript{His} was recovered on the beads (Figure 5A).

Western blotting of both the Ni-NTA purified and unbound material indicated that the recovery of Oxa1\textsubscript{His} on the beads was complete (Figure 5C). Approximately 5% of the Atp5 protein was recovered with Oxa1\textsubscript{His} on the Ni-NTA beads, thus giving an indication of the level of ATP synthase complexes that can be found in association with Oxa1 under these purification conditions (Figure 5C). Our findings here suggest that the ATP synthase complex purified together with Oxa1\textsubscript{His} may represent an assembled F\textsubscript{\textgamma}F\textsubscript{\textalpha}-complex. Analysis of the electrophoretic profile of the subunits, which copurified with Oxa1\textsubscript{His}, was similar to that of the ATP synthase complex purified in parallel under the same Triton X-100 lysis conditions by using the His-tagged version of the \beta-subunit of the F\textsubscript{\textgamma}-sector (Mueller \textit{et al.}, 2004; Figure 5D), suggesting that an intact or very late assembly intermediate of the ATP synthase complex was associated with Oxa1\textsubscript{His}.

The recovery of the ATP synthase components with the Oxa1\textsubscript{His} protein was possibly due to its prior assembly into an SDS-resistant complex between Oxa1 and newly synthesized Atp9 (Figure 5E). As shown previously (Figure 4B), oligomycin also appears to affect the ability of newly synthesized Atp9 and Atp6 proteins to assemble together in a stable manner. Pretreatment of mitochondria with carbonyl cyanide m-chlorophenylhydrazone (CCCP) before detergent lysis and NiNTA purification, did not adversely affect the ability of the Oxa1 protein to remain in association with the ATP synthase complex (results not shown). CCCP is a potent dissipater of the proton motive force and hence indirectly inhibits the F\textsubscript{\textgamma}F\textsubscript{\textalpha}-ATP synthase, because of the lack of an available pH gradient to drive the forward ATP synthesis reaction. We therefore consider it likely that the ATP synthase-specific inhibitor oligomycin interferes with the F\textsubscript{\textgamma}-sector–Oxa1 interface directly, rather than indirectly through an inhibition of the ATP synthesis activity of the F\textsubscript{\textgamma}F\textsubscript{\textalpha}-complex. In support of this conclusion, formation of the previously described SDS-resistant complex between Oxa1 and newly synthesized radiolabeled Atp9 was also adversely affected when the in organello translation reaction was performed in the presence of oligomycin (Figure 5F). Formation of the Atp9–Oxa1 complex was not affected when translation was performed in the presence of CCCP or valinomycin (dissipates the \Delta\psi aspect of proton motive force; Figure 5F). These results support the conclusion that oligomycin acts directly on the Atp9–Oxa1 interface to disrupt this interaction and is not acting indirectly through modulation of the electrochemical gradient and hence the activity of the ATP synthase enzyme.

In summary, we demonstrate that Oxa1 can physically interact with the assembled ATP synthase complex in a specific manner. Furthermore, association of Oxa1 with the ATP synthase appears to involve binding at the Atp9 interface because, like the newly synthesized Atp9–Oxa1 interaction, it is perturbed by the presence of oligomycin. It is important to note that there are multiple copies of Atp9 subunit and only one Atp6 subunit per F\textsubscript{\textgamma}-sector. It is therefore conceivable that in a given F\textsubscript{\textgamma}-sector Atp9–Oxa1 and Atp9–Atp6 interactions may occur, and this may explain why Atp6 is copurified with the other ATP synthase subunits bound to the Oxa1\textsubscript{His} protein.

**Association of Oxa1 with Newly Synthesized Atp9 Can Occur before It Assembles into an F\textsubscript{\textgamma}F\textsubscript{\textalpha}-ATP Synthase Complex**

Given that Oxa1 can associate with the assembled ATP synthase complex, we next addressed whether the observed association between the newly synthesized Atp9 and the Oxa1 protein was possibly due to its prior assembly into an assembled F\textsubscript{\textgamma}F\textsubscript{\textalpha}-ATP synthase complex or whether Atp9’s association with Oxa1 occurs before the complete assembly of Atp9. We therefore compared the amount of newly synthesized Atp9, which can be found in association with Oxa1\textsubscript{His} with that associated with the F\textsubscript{\textgamma}-ATP synthase complex, using mitochondria harboring the Atp9\textsubscript{His} derivative (Figure 6). Association of radiolabeled Atp9 with the Atp9\textsubscript{His} protein would be observed if the in organello synthesized Atp9 protein can assemble with the F\textsubscript{\textgamma}-sector after synthesis in isolated mitochondria.

In organello translation in the presence of [\textsuperscript{35}S]methionine was performed in mitochondria bearing the Oxa1\textsubscript{His} protein or Atp\beta\textsubscript{His} protein and after translation, mitochondria were lysed in buffer containing Triton X-100 (detergent conditions that maintain the assembled F\textsubscript{\textgamma}F\textsubscript{\textalpha}-ATP synthase complex intact) and the solubilized proteins were subjected to NiNTA chromatography. When translation was performed in the mitochondria bearing the Oxa1\textsubscript{His} derivative, both the SDS-resistant Oxa1\textsubscript{His}–Atp9 72-kDa complex and monomeric Atp9 were recovered on the Ni-NTA beads, as previously described (Figure 6, right panels). The level of newly
synthesized Atp9 found in association with the AtpβHis protein, however, was significantly lower than that observed together with the Oxa1His protein (Figure 6, right panels), indicating that the majority of the radiolabeled Atp9 purified with Oxa1His had not been assembled into an F1F0-ATP synthase complex. On the other hand, immunedecoration with Atp9-specific antiserum showed that significantly more of the endogenous (i.e., pre-existing/assembled) Atp9 was recovered with the purified AtpβHis protein, than with the Oxa1His protein (Figure 6, left panel). A similar observation was made also for the preexisting Atp6 protein (Figure 6, left panel). The recovery of the Atp9 and Atp6 proteins with the AtpβHis protein further confirms the ability of this protein to affinity-purify the assembled ATP synthase complex, as was previously indicated in Figure 5D. It is important also to note here that the radiolabeled Atp9 protein affinity-purified with AtpβHis and Oxa1His comigrated with the immunedecorated endogenous Atp9 protein using a gel system where Atp9 can be resolved from Atp8, thus providing further verification for the identity of the radiolabeled Atp9 protein associated with Oxa1 after synthesis in isolated mitochondria.

**Newly Synthesized Atp9 Can Associate with C-Terminaly Truncated Oxa1**

Finally, we investigated whether the C-terminal matrix located region of Oxa1 is not required for the Atp9-Oxa1 interaction. In organello translation was performed in yeast strains expressing Oxa1, Oxa1His, or Oxa1Δ83His derivatives under control of the GAL10 promoter. (A) In organello translation in the presence of [35S]methionine was performed in the mitochondrion, and samples were further analyzed as described in Figure 6. (B) Immunoblotting of Atp5 was performed, in addition to Atp6 and Atp9, and the presence of Oxa1His and Oxa1Δ83His on the Ni-NTA beads were detected using an Oxa1 antibody specific for the N-terminal region of Oxa1.

The C-terminal region of Oxa1 is not required for the Atp9-Oxa1 interaction. (A and B) Mitochondria were isolated from yeast strains expressing Oxa1, Oxa1His, or Oxa1Δ83His derivatives, and samples were further analyzed as described in Figure 6. (B) Immunoblotting of Atp5 was performed, in addition to Atp6 and Atp9, and the presence of Oxa1His and Oxa1Δ83His on the Ni-NTA beads were detected using an Oxa1 antibody specific for the N-terminal region of Oxa1.
stronly reduced relative to Oxa1 \(_{\text{ts}}\) and this accounts for the reduced level of Oxa1\(_{\Delta 3\text{his}}\) which was recovered quantitatively, on the Ni-NTA beads (results not shown). Thus we conclude the reduced level of radiolabeled and assembled Atp9 recovered with the Oxa1\(_{\Delta 3\text{his}}\) derivative reflects the lowered levels of Oxa1\(_{\Delta 3\text{his}}\) present in the mitochondria, rather than an impaired affinity of the C-terminally truncated Oxa1 for the Atp9 protein. It would appear therefore that the addition of the C-terminal histidine tag affected the stability of the Oxa1\(_{\Delta 3\text{his}}\) derivative, because previous studies have shown that the levels of the Oxa1\(_{\Delta 3}\) protein, i.e., nontagged, can be comparable to the authentic Oxa1 protein (Jia et al., 2003; Szyrach et al., 2003).

Taken together, we conclude that the C-terminal region of Oxa1 is not required for the observed association between Oxa1 and the newly synthesized Atp9 or the endogenous ATP synthase components.

**DISCUSSION**

We report here that Oxa1 associates with and supports the assembly of the \(F_1\)-sector ATP synthase complex in yeast mitochondria. In the absence of Oxa1, a strong decrease in the levels of \(F_1\)-sector ATPase enzyme activity was observed, and this decrease corresponds to a reduction in the overall protein levels of the assembled \(F_1\)-sector ATP synthase complex. The severely reduced levels of \(F_1\)-sector ATP synthase complex observed in the \(oxa1\) mitochondria appear to be directly due to the absence of Oxa1 and are not due to an indirect consequence of the absence of the assembled COX complex in these mitochondria.

It is important to note, however, that although strongly reduced in levels, assembled and enzymatically functional ATP synthase complexes can still be detected in the \(oxa1\) mitochondria, suggesting that a low level of Oxa1-independent ATP synthase assembly can occur. This is in contrast to the COX complex where an essential role for Oxa1 in facilitating the insertion of mitochondrial encoded subunits, Cox1 and Cox2, is well documented. Our findings here, however, do not support a role for the Oxa1-related protein, Cox18, in the assembly of the ATP synthase complex, even in the absence of Oxa1. We conclude therefore that although the presence of Oxa1 is important for the biogenesis of the \(F_1\)-sector ATP synthase complex, it is clearly not essential and that level of Oxa1-, Cox18-independent assembly of this complex can occur. Cox18 has been proposed to play a role in the posttranslational insertion of the C-terminal region of Cox2, which becomes inserted into the membrane with two transmembrane segments (TMs) to attain a \(N_{\text{out}}C_{\text{out}}\) orientation. Translocation of the C-terminal domain of Cox2 (~150 residues) to the intermembrane space ensues in a posttranslational manner. Atp9 also contains two TM segments and attains a \(N_{\text{out}}C_{\text{out}}\) membrane topology similar to that of Cox2 (but does not contain an extended hydrophilic intermembrane space exposed C-terminal tail) but clearly does not depend on Cox18 for its insertion of assembly events. We conclude therefore that Cox18 does not represent a common posttranslational translocase for all mitochondrial encoded proteins.

We demonstrate here that Oxa1 can directly associate with the newly synthesized Atp9 protein before its assembly into an intact \(F_1\)-sector ATP synthase complex. Furthermore, the observed interaction of Atp9 with Oxa1 does not require the matrix-localized C-terminal, basic tail of Oxa1, a region of the protein that is limited to the Oxa1-family members, i.e., not conserved among the Cox18/Oxa2 proteins. Thus the presence of the C-terminal tail region of Oxa1 does not account for the reason that Oxa1, but not Cox18, is required for the assembly of Atp9 into the ATP synthase complex, but rather may reflect some sequence specific differences in their transmembrane segments or N-terminal regions. It is important to note that the observed physical association of Oxa1 with newly synthesized Atp9 in a posttranslational manner is in marked contrast to that previously observed for Oxa1 and its COX subunit substrates, the Cox1, Cox2, and Cox3 subunits, which transiently interact with Oxa1 and only in a cotranslational manner. After completion of synthesis, the full-length, membrane-inserted COX subunits do not remain in association with Oxa1. It has been previously suggested that proteins that coordinate events downstream of membrane insertion, i.e., the subsequent assembly of membrane-inserted polypeptides, are more likely to bind to these proteins in a posttranslational and stable manner (Tzagoloff et al., 2004). Binding of assembly proteins or chaperones to substrate proteins in this manner is argued to maintain the competence of their substrates for subsequent assembly events. We propose therefore that the observed posttranslational and stable association between Atp9 and Oxa1 indicates a postmembrane insertion role for Oxa1 in the assembly of Atp9 into the \(F_1\)-sector.

Consistently, the results presented here demonstrate the ability of Atp9 to assemble into its oligomeric form and to associate with \(F_1\)-sector subunits in the absence of Oxa1. Indeed the size exclusion chromatography experiments demonstrate that the Atp9-F\(_1\)-subcomplex present in the \(oxa1\) mitochondria is similar to those in the \(aotp10\) strain, a mutant reported to be defective in the step of integration of the Atp6 protein into late Atp9-F\(_1\)-assembly intermediates (Tzagoloff et al., 2004). Taken together these data support a model where the activity of Oxa1 is required downstream of the translation and membrane insertion events for the mitochondrial encoded Atp9 subunit. The involvement of Oxa1 in the assembly of the mitochondrial \(F_1\)-sector ATP synthase complex may differ therefore from that reported for the YidC (the prokaryotic homolog of Oxa1) and the bacterial ATP synthase complex. Current evidence supports a role of YidC in the membrane insertion step of ATP synthase subunit c (the prokaryotic equivalent of Atp9; van Bloois et al., 2004; van der Laan et al., 2005; Kol et al., 2006). On the basis of the demonstrated posttranslational interaction between newly synthesized Atp9 and Oxa1 and the ability of Atp9 to assemble into the Atp9-F\(_1\) subcomplex in the absence of Oxa1, we propose that the mitochondrial Oxa1 acts in a postmembrane insertion step for Atp9. Furthermore, from the data presented it appears that the assembly of Atp6 into the accumulated Atp9-F\(_1\)-subcomplex represents the step that is adversely affected in the \(oxa1\) mitochondria.

By interacting with the newly synthesized Atp9 oligomer in a posttranslational manner, Oxa1 may serve to maintain the Atp9 oligomer in a “competent state” to coassemble with the Atp10-chaperoned Atp6 protein. Thus in the absence of either Oxa1 or Atp10, the efficient assembly of the Atp6-Atp9 containing \(F_1\)-sector, would be hindered, due to a lack of Atp9- or Atp6-specific chaperones, respectively. Alternatively, it is possible that in the absence of Oxa1, the problem may lie at the level of the newly synthesized Atp6 protein, for example, that it fails to become efficiently inserted into the inner membrane and hence does not associate with, an otherwise competent Atp9 oligomer. Available data, however, would favor that insertion of Atp6 is not severely blocked when the translocase activity of Oxa1 is compromised. The insertion of newly synthesized Atp6 was only partially affected (decreased by ~35%, relative to wild type) in the pet ts1402 oxa1 mutant mitochondria, conditions
where the translocase activity of Oxa1 was severely affected, as judged by inhibition of Cox2 insertion (which was ~95% inhibited; Hell et al., 2001). For these reasons we favor a role for Oxa1 in the assembly of the Fₚ-sector of the ATP synthase is related to its ability to associate with Atp9 in a posttranslational manner. We propose that features of Oxa1 distinct from those required for its translocation activity of the COX subunits, are necessary to chaperone the newly synthesized Atp9 protein and by doing so support its assembly with Atp6.

A dichotomy in Oxa1’s functions therefore appears to exist, whereby the translocase activity of Oxa1 required for the COX subunits, is distinct from another function of Oxa1 that is required in the assembly of Atp9 into the FₚFₒ-ATP synthase complex. In support of this proposal, an oxa1 mutant, bearing a mutation in the first TM region of Oxa1 has been reported to significantly affect cytochrome oxidase formation, whereas the assembly of the ATP synthase complex was relatively unaffected (Lemaire et al., 2004). Indeed other oxa1 mutants, which bear mutations within the intermembrane space exposed loop region between the second and third transmembrane segments of the Oxa1 protein, also display a strong reduction in COX levels and yet have normal assembly levels of the FₚFₒ-ATP synthase complex (Bushman and Stuart, unpublished results). Taking this together with the data reported here, we argue that the translocase activity of Oxa1 is not directly required for the Fₚ-sector subunits of the mitochondrial ATP synthase complex.

We report here also that a subpopulation of the ATP synthase complex can be copurified with a C-terminal His-tagged Oxa1 protein. The subunit profile of the ATP synthase complex which was found in association with Oxa1₃His was very similar to that affinity purified directly using a His-tagged Atp9 subunit. These findings suggest that an almost- or completely assembled ATP synthase complex can exist in physical association with the Oxa1 protein. The binding of Oxa1 to the ATP synthase complex most likely involves the Fₚ-sector and more specifically occurs at the ATP9 interface, because the copurification of these complexes was sensitive to pretreatment of the mitochondria with oligomycin before the detergent lysis step. The observed coassociation of ATP synthase with the Oxa1₃His however, was not artificially caused by the elevated levels of the Oxa1 derivative in these mitochondria (expressed under the control of GAL10 promoter and hence levels of Oxa1 were ~50 times greater than endogenous levels; results not shown). A similar copurification of FₚFₒ-ATP synthase subunits was also observed when the Oxa1₃His derivative was expressed at endogenous levels under control to Oxa1’s own promoter region. Under these conditions, significantly lower levels of ATP synthase subunits were, however, recovered with Oxa1₃His on the Ni-NTA beads, but these levels paralleled the lower content of the Oxa1₃His protein in the sample (result not shown).

Why does Oxa1 associate with the ATP synthase complex and does this interaction have any bearing on the role of Oxa1 in the biogenesis of the ATP synthase complex? The ability of Oxa1 to bind to the assembled ATP synthase complex, most likely reflects the innate ability of Oxa1 to interact with the Atp9 protein. Common features of the newly synthesized Atp9 protein and the oligomeric Atp9 assembled in the FₚFₒ-ATP synthase, appears to support the observed Oxa1 associations, because both interactions are sensitive to the pretreatment with oligomycin. The ability of the ATP synthase to physically interact with another inner membrane protein complex is not unique to Oxa1. It has been previously described that the FₚFₒ-ATP synthase complex can be found with the Mdh1 protein, an Atp-binding cassette transporter (ABC) protein, involved in peptide export across the inner membrane (Galluhn and Langer, 2004). We did not observe association of Mdh1 with the Oxa1₃His protein under the lysis conditions used in this study (results not shown).

It is presently unclear from our studies whether the observed association between these complexes is for the benefit of Oxa1 or of the ATP synthase complex (or possibly even for their mutual benefit). The Oxa1-ATP synthase association may be to support the activity of Oxa1, either directly in a bioenergetic manner or indirectly to regulate the activity of Oxa1 as a translocase for the COX subunits, possibly to maintain a balance between the biogenesis levels of COX and ATP synthase complexes. It is equally plausible that the Oxa1-ATP synthase association serves to benefit the ATP synthase complex during its final steps of assembly. The association of Oxa1 with the Atp9 may interfere with the H⁺ pumping activity of the complex, because the oligomycin sensitivity of binding may indicate that Oxa1 binds at the interface of the Atp6/Atp9 proteins. The stable association of Oxa1 with the Fₚ-sector in this manner may ensure that the ATP synthase complex remains inactive (i.e., not leaky to H⁺ transfer, which may dissipate of the membrane potential) until the complete assembly of a tightly coupled FₚFₒ-ATP synthase complex is achieved. At any given time therefore, a small percentage of mitochondrial ATP synthase complexes, completing their assembly may be found in association with Oxa1, their assembly chaperone.

In summary, we demonstrate here an important role for Oxa1, but not Cox18, in the assembly of the FₚFₒ-ATP synthase complex. Oxa1 directly interacts with Atp9 after its translation in mitochondria, and we propose that this interaction with Oxa1 is necessary to chaperone Atp9 and ensure the efficient and correct assembly into an active and coupled FₚFₒ-ATP synthase complex. Further experiments to investigate the molecular basis for the physiological relevance of the observed Oxa1-ATP synthase association are currently underway in our laboratory.

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REFERENCES


van Bloois, E., Haan, G. J., de Gier, J. W., Ouodega, B., and Luirink, J. (2004). F_{1}F_{0} ATP synthase subunit c is targeted by the SRP to YidC in the E. coli inner membrane. FEBS Lett. 576, 97–100.


