

Marquette University

e-Publications@Marquette

Biological Sciences Faculty Research and
Publications

Biological Sciences, Department of

5-2007

Oxa1 Directly Interacts with Atp9 and Mediates Its Assembly into the Mitochondrial F₁F₀-ATP Synthase Complex

Lixia Jia
Marquette University

Mary Kathryn Dienhart
Marquette University

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

Follow this and additional works at: https://epublications.marquette.edu/bio_fac



Part of the [Biology Commons](#)

Recommended Citation

Jia, Lixia; Dienhart, Mary Kathryn; and Stuart, Rosemary A., "Oxa1 Directly Interacts with Atp9 and Mediates Its Assembly into the Mitochondrial F₁F₀-ATP Synthase Complex" (2007). *Biological Sciences Faculty Research and Publications*. 365.

https://epublications.marquette.edu/bio_fac/365

Oxa1 Directly Interacts with Atp9 and Mediates Its Assembly into the Mitochondrial F₁F_o-ATP Synthase Complex

Lixia Jia, Mary K. Dienhart, and Rosemary A. Stuart

Department of Biological Sciences, Marquette University, Milwaukee, WI 53233

Submitted October 18, 2006; Revised January 29, 2007; Accepted February 26, 2007

Monitoring Editor: Janet Shaw

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 F₁F_o-ATP synthase complex. We demonstrate that Oxa1, but not Cox18/Oxa2, directly supports the assembly of the membrane embedded F_o-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 F_o-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 1; 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 ($\Delta oxa1$) 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 *bc*₁ and F₁F_o-ATP synthase complexes are significantly reduced in the $\Delta oxa1$ mitochondria (Altamura *et al.*, 1996; Szyrach *et al.*, 2003). However, in contrast to the COX complex, which is completely absent in $\Delta oxa1$ mitochondria, the cytochrome *bc*₁ complex and F₁F_o-ATP synthase complex activities, although strongly reduced, are clearly still detectable in $\Delta oxa1$ 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 *bc*₁ and ATP synthase enzymes in $\Delta oxa1$ 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

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-10-0925>) on March 7, 2007.

Address correspondence to: Rosemary A. Stuart (rosemary.stuart@marquette.edu).

proteins including subunits of the cytochrome oxidase (CyoA) and subunit c (equivalent to mitochondrial Atp9) of the F_1F_o -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 *Neurospora 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_1F_o -ATP synthase complex can be divided into two functionally distinct parts, the hydrophilic F_1 -part, which performs the ATP synthesis and hydrolysis events, and the membrane-embedded F_o -sector, which facilitates the passage of protons across the inner membrane. The F_1 -sector is composed entirely of nuclear-encoded proteins, whereas the F_o -complex is assembled from both nuclear and mitochondrially encoded proteins. The mitochondrially encoded Atp6, Atp8, and Atp9 are all subunits of the F_o -sector. The Atp9 subunit spans the inner membrane twice in an N_{out} - C_{out} 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_1 -sector (Stock *et al.*, 1999; Devenish *et al.*, 2000; Velours and Arselin, 2000; Capaldi and Aggeler, 2002). During the assembly of the ATP synthase complex, the newly synthesized Atp9 has been shown to oligomerize and form an F_1 -containing subcomplex before associating with the Atp6 protein (see Ackerman and Tzagoloff, 2005 for recent review). The assembly of Atp6 with the F_1 -Atp9 subcomplex thus represents a late step in the assembly pathway of the F_1F_o -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_1 -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_o -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_o -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_1F_o -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*), ρ^0 W303-1A (Mata, *leu2*, *trp1*, *ura3*, *his3*, *ade2*), and the *oxa1*-null mutant, $\Delta oxa1$ (W303-1A, *leu2*, *trp1*, *ura3*, *ade2*, *OXA1::KAN^r*), whereby the complete *OXA1* open reading frame (ORF) was replaced by the kanamycin resistance gene (*KAN^r*) using homologous recombination (Wach *et al.*, 1994). Construction of the strains $\Delta cox18$ and $\Delta oxa1$, $\Delta cox18$ was performed by the introduction of the *HIS3* gene into the *COX18* locus of wild-type and $\Delta oxa1::KAN^r$ cells, resulting in the replacement of the entire *COX18* ORF. Correct homologous recombination of the *HIS3* or *KAN^r* gene at their respective gene locus was verified by PCR analysis (results not shown). Construction of the $\Delta imp1$ strain was performed by introduction of 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_{His} 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 (Oxa1 $\Delta 83_{His}$) 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-*OXA1* $\Delta 83_{His}$ was cloned into the yeast genome of the *oxa1*-null mutant at the *LEU2* locus after linearization with *Bst*II.

H₂O₂ Treatment and Dispersal of Atp9 Oligomer to Monomers

Mitochondria (200 μ g) in 20 mM Tris-HCl, pH 8.0, buffer were mixed with H₂O₂, 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 H₂O₂ 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_1F_o -ATP synthase complexes after solubilization with digitonin was performed essentially as described previously (Wittig and Schagger, 2005). Mitochondria (200 μ g of protein) were lysed with 36 μ l 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 \times g). The supernatants were collected, and 2 μ l 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_1F_o -ATPase activity assay was performed after CN-PAGE, as previously described (Wittig and Schagger, 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 μ 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 MgSO_4 , 0.2% $\text{Pb}(\text{NO}_3)_2$, 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 Oxa1_{His} Complex

Mitochondria harboring the GAL10 expressed Oxa1_{His} derivative (200 μ g) were solubilized in 200 μ 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 (20,860 $\times g$, 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) β -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 μ g protein) were solubilized in 200 μ 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 Atp β .

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 Δoxa1 and Δcox18 and also from the double gene knockout strain Δoxa1 , Δcox18 were solubilized with the detergent digitonin, and the resulting extracts were subjected to clear 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 Δimp1 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

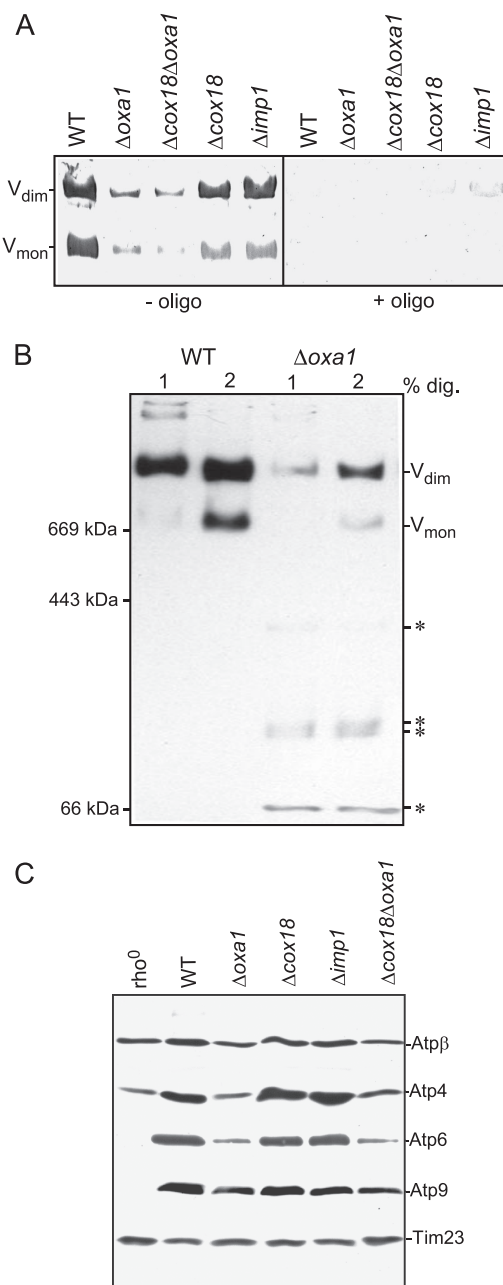


Figure 1. Characterization of assembly defects of ATP synthase in Δoxa1 and Δcox18 mutants. (A) Mitochondria from wild-type (WT), Δoxa1 , Δcox18 , Δoxa1 , Δcox18 , and Δimp1 strains were solubilized with buffer containing 2% digitonin and subjected to CN-PAGE followed by in-gel ATPase activity assay in the absence or presence of oligomycin (5 μ 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 Δoxa1 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 Δoxa1 mitochondria are indicated by an asterisk (*). (C) Mitochondria from rho⁰, WT, Δoxa1 , Δcox18 , Δimp1 , and Δcox18 , Δoxa1 strains were subjected to SDS-PAGE and Western blotted using antisera against F₁ subunit β (Atp β), F₀ 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.

dimers by the increased detergent concentrations (Arnold *et al.*, 1998). Enzymatically active F_1F_o -ATPase complexes were also detected in the $\Delta oxa1$ mitochondrial extract. However, the levels of these complexes were strongly reduced when compared with that in wild-type mitochondria (~20% of wild-type levels; Figure 1A). Observation of both dimeric and monomeric forms of the complex in the $\Delta oxa1$ mitochondria indicated that the ATP synthase complexes could fully assemble, albeit at reduced levels, in the absence of Oxa1. Importantly, the F_1F_o -ATP synthase complex in $\Delta oxa1$ mitochondria could also be inhibited through the addition of oligomycin (Figure 1A). This inhibition is an indicator of the correct and functional assembly of the coupled F_1F_o -complexes in the $\Delta oxa1$ mitochondria, because oligomycin acts specifically on the F_o -sector, where it is thought to bind to the region of Atp9 that interfaces with Atp6 (John and Nagley, 1986; Nagley *et al.*, 1986). Uncoupled F_1 -complexes are not inhibited through the addition of oligomycin. In the $\Delta oxa1$ mitochondria, a low level of smaller molecular weight complexes displaying oligomycin-insensitive ATPase activity were also detected, indicating the possible presence of partially assembled F_1 -intermediates in the absence of Oxa1 (results not shown).

Enzymatically active F_1F_o -ATP synthase complexes, which were oligomycin sensitive, were clearly detected in the $\Delta cox18$ mutant at the levels that were only slightly reduced when compared with the wild-type control mitochondria. Indeed the levels of active ATP synthase complexes in the $\Delta cox18$ mitochondria were similar to those of an independent COX assembly mutant, the $\Delta imp1$ strain (Figure 1A). Imp1 is a protease involved in the maturation of the Cox2 protein, and $\Delta imp1$ mutants are deficient in COX complexes (Nunnari *et al.*, 1993). Subunits of the ATP synthase complex do not undergo Imp1-specific proteolytic events; hence, the assembly of this complex is not directly affected in the $\Delta imp1$ mutants. The slight reduction of ATP synthase levels in the $\Delta cox18$ and $\Delta imp1$ mutants may therefore be due to an indirect effect in response to the absence of the COX complex activity (Figure 1A).

The activity levels of the ATP synthase complex, and its sensitivity to oligomycin, in the $\Delta cox18$, $\Delta oxa1$ mitochondrial were comparable to those isolated from the $\Delta oxa1$ strain (Figure 1A). Thus, despite sharing sequence homology, the Cox18 and Oxa1 proteins do not appear to bear overlapping functions with regards to the assembly of the ATP synthase complex. Deletion of the COX18 gene did not abolish the ability of a significant, albeit reduced, level of F_1F_o -ATP synthase complex to assemble in the absence of Oxa1.

To demonstrate that the strongly reduced levels of enzymatically active ATP synthase complexes in the $\Delta oxa1$ mitochondria reflected an overall reduction in the protein levels of the complex, rather than a lack or inhibition of its activity, the protein levels of the assembled ATP synthase in $\Delta oxa1$ mitochondria was further analyzed after CN-PAGE analysis by Western blotting and immunodecoration with antisera against the purified F_1 -complex (Figure 1B). Consistent with the enzyme activity measurements, the level of the assembled ATP synthase dimers and monomers were significantly reduced, but remained detectable in the $\Delta oxa1$ mitochondria (Figure 1B). Furthermore in the $\Delta oxa1$ mitochondria, lower molecular weight F_1 -complexes, corresponding to partially assembled F_1 -containing complexes were also observed, indicating a defect in the assembly of the ATP synthase complexes in the absence of Oxa1. This level of partially assembled F_1 -complexes was not observed in the wild-type control mitochondria. A similar accumulation of partially assembled F_1 -complexes was also observed in the $\Delta oxa1$, $\Delta cox18$

double mutant, but not, however, in the $\Delta cox18$ single null mutant mitochondria (results not shown).

Taken together, the CN-PAGE results indicate a strong reduction in both the protein and enzyme activity levels of the assembled F_1F_o -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 F_1F_o -ATP synthase complex.

F_o -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 F_o -sector in $\Delta oxa1$ mitochondria. Mitochondria isolated from the single null mutant strains, $\Delta oxa1$ and $\Delta cox18$ and also from the double knockout strain $\Delta cox18$, $\Delta oxa1$ were analyzed by SDS-PAGE and Western blotting. Wild-type, COX-deficient $\Delta imp1$ and rho⁰ (contain no mtDNA) mitochondria were also analyzed in parallel as controls. Immunodecoration was subsequently performed to assess the steady state levels of the F_o -sector subunits Atp4, Atp6, and Atp9 and also the F_1 -sector subunit, Atp β (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 $\Delta cox18$ mitochondria, because they were similar to those observed in the wild-type and $\Delta imp1$ mitochondria. In the $\Delta oxa1$ 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 $\Delta oxa1$ strain were not so dramatically affected as those of Atp6 or Atp4 proteins. The levels of Atp4, Atp6, and Atp9 in the $\Delta cox18$, $\Delta oxa1$ double mutant were similar to those of the $\Delta oxa1$ 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 F_o -ATP synthase subunit, Atp8, because an antibody specific for the Atp8 protein was not available. In contrast to the F_o -subunits, the F_1 -sector subunit Atp β was not so significantly reduced in the $\Delta oxa1$ mitochondria. Thus it would appear that the reduction in assembled F_1F_o -ATP synthase complexes in the absence of Oxa1 reflects the limiting levels of F_o -sector, rather than F_1 -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 F_o -sector of the ATP synthase complex in a posttranslational manner and before their assembly into the F_1F_o -ATP synthase complex. Three of the F_o -sector subunits are encoded by the mitochondrial DNA: Atp6, Atp8, and Atp9. To address a possible association between Oxa1 and these subunits of the F_o -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 Oxa1_{His}-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

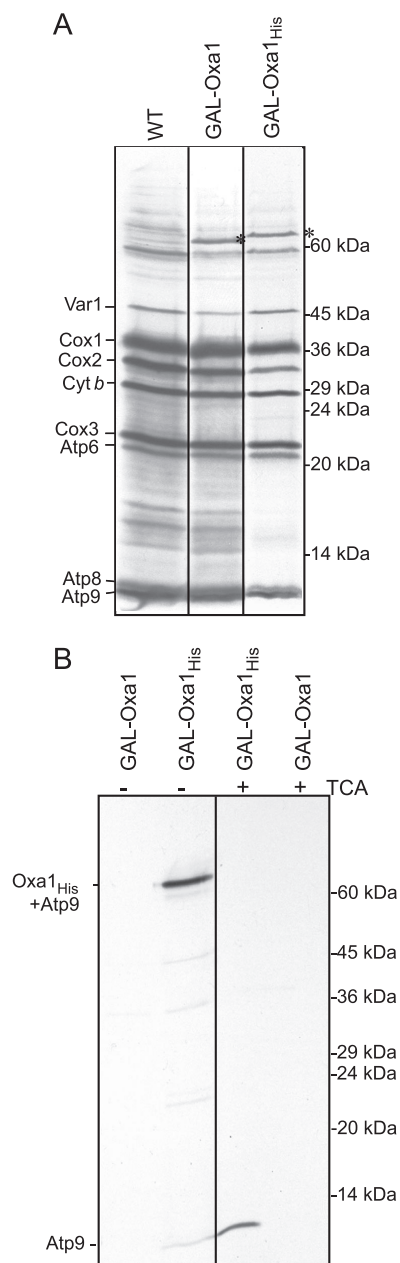


Figure 2. Oxa1 associates specifically with newly synthesized Atp9. (A) In organello translation was performed in the presence of [35 S]methionine for 30 min at 25°C in mitochondria isolated from the wild-type (WT) yeast strain, a strain overexpressing Oxa1 (GAL-Oxa1) and from a strain expressing histidine-tagged 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 mitochondrial-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 product(s) 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.

forms of the mitochondrially 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 mitochondrially 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-containing 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 F₀-subunits, appear not to be drastically reduced in the absence of Oxa1. We hypothesized therefore that Atp9 can assemble to an intermediate

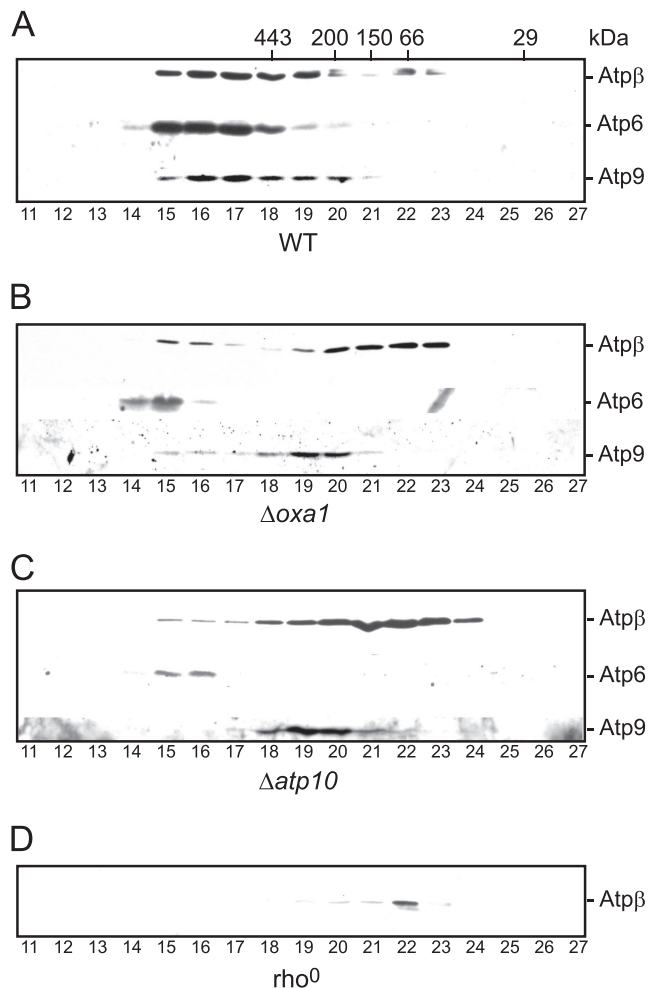


Figure 3. Atp9 assembles into an F_1 -containing subcomplex in the absence of Oxa1. Mitochondria from wild-type (WT; A), $\Delta oxa1$ (B), $\Delta atp10$ (C), and ρ^0 (D) strains were solubilized with 1% Triton X-100 containing buffer and subjected to size exclusion chromatography on Superose 12 column. Fractions (11–27) eluted from the column were TCA precipitated and subjected to SDS-PAGE followed by Western blotting with antisera against F_1 subunit Atp β and F_0 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 $\Delta oxa1$ and $\Delta atp10$ mitochondria was significantly longer than that for the wild-type control, because of the strongly decreased levels of Atp6 in these mutant mitochondria.

stage and be at least partially stabilized in the absence of Oxa1.

We therefore addressed the assembly state of the endogenous Atp9 in the $\Delta oxa1$ mitochondria and compared it to that of Atp9 accumulated in the $\Delta atp10$ mitochondria (Figure 3). Wild-type and ρ^0 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 F_1F_0 -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 Atp β , in fractions 15–18, corresponding to a complex of ~550 kDa. Some Atp9 eluted in later fractions together with a low level of Atp β

(fractions 19 and 20), with a molecular mass of ~300 kDa, and most likely corresponds to the previously described Atp9- F_1 subcomplex. Atp6 was found exclusively with the fully assembled ATP synthase complex, however (Figure 3A).

In the $\Delta oxa1$ mitochondria, the majority of Atp9 eluted from the column with some of the Atp β protein, in fractions 19 and 20, which correspond to the ~300-kDa Atp9- F_1 subcomplex. Only a minor fraction of Atp9 eluted from the column in the fractions (fractions 15–18) corresponding to fully assembled F_1F_0 -ATP synthase, as indicated by the coelution of Atp6 and Atp β (Figure 3B). The observed low level of fully assembled F_1F_0 -ATP synthase complexes in the $\Delta oxa1$ strain is consistent with the previous CN-PAGE results (Figure 1). Hence only a small fraction of the total Atp β subunit was present in the fractions corresponding to the fully assembled F_1F_0 -ATP synthase complex; the majority of Atp β eluted from the column either with Atp9- F_1 subcomplex (fractions 19 and 20) or later in F_1 -complex of ~150 kDa (fractions 21–23). The latter F_1 -complex was similar in mass as an Atp β -containing assembly intermediate that could be observed in the ρ^0 mitochondria, which lack all of the mtDNA-encoded F_0 -sector subunits, Atp9, Atp8, and Atp6 (Figure 3D). We conclude from these results that in the $\Delta oxa1$ mitochondria, the Atp9 protein, which does not assemble into the complete F_1F_0 -ATP synthase complex, accumulates in an Atp9- F_1 subcomplex. The behavior of the Atp9 in the $\Delta oxa1$ mitochondria was similar to that in the $\Delta atp10$ 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 F_1 -subunits, occurs in an Atp10-independent manner; but further assembly of this oligomer with Atp6 does not occur in the absence of Atp10, thus preventing the complete assembly of the F_1F_0 -ATP synthase complex. The similarity of the behavior of Atp9 in $\Delta oxa1$ and the $\Delta atp10$ mitochondria, i.e., competence to assemble in a stable

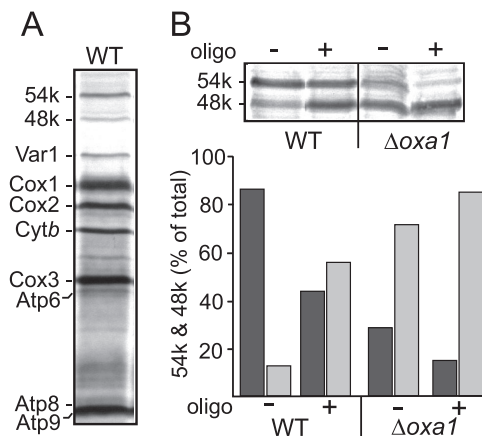


Figure 4. Assembly of the ATP synthase complex is impaired in $\Delta oxa1$ mitochondria. Mitochondria from wild type (A and B) and $\Delta oxa1$ (B) strains were subjected to translation and radiolabeled for 30 min at 25°C followed by a chase period in the presence of excess cold methionine, an ATP-regenerating system and in the absence or presence of oligomycin, as indicated. Mitochondria were reisolated, lysed and subjected to SDS-PAGE, Western blotting, and autoradiography. The positions of the 54-kDa SDS-resistant complex between Atp9 and Atp6, 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.

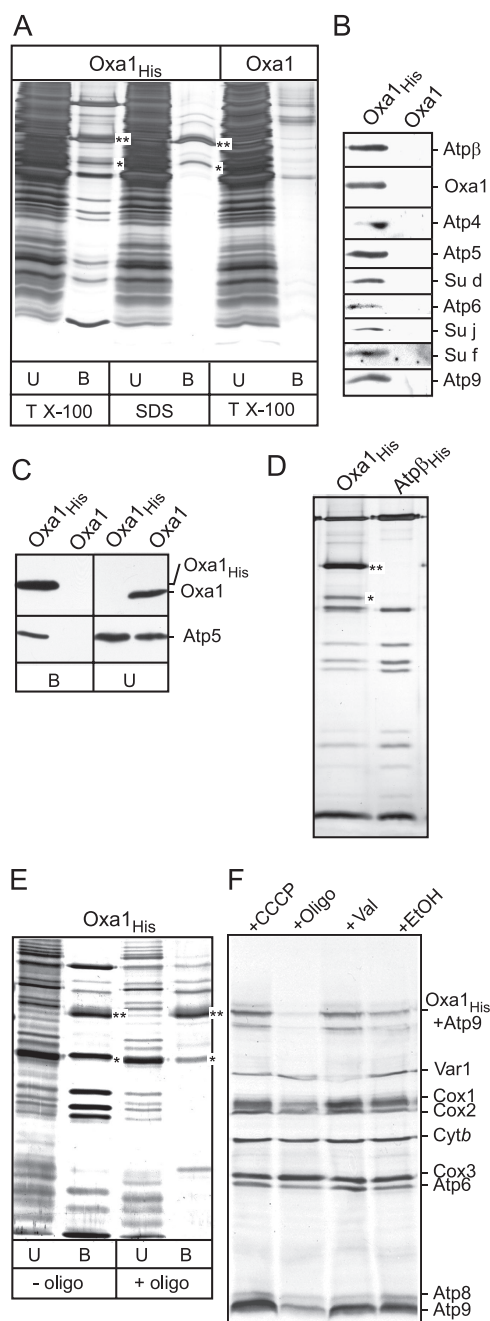


Figure 5. The ATP synthase complex can be copurified together with Oxa1. (A) Mitochondria isolated from strains expressing Oxa1_{His} or Oxa1 were solubilized with buffer containing either 1% Triton X-100 (TX-100) or 0.1% SDS as indicated and incubated with Ni-NTA beads as described in *Materials and Methods*. Twenty percent of the unbound material (U) and the Ni-NTA beads purified material (indicated by B) were analyzed by SDS-PAGE and silver-staining. (B) Mitochondria isolated from strains expressing Oxa1_{His} or Oxa1 (GAL-Oxa1 and GAL-Oxa1_{His}, respectively) were solubilized with buffer containing 1% Triton X-100, as indicated, and were incubated with Ni-NTA beads. The Ni-NTA-purified material was analyzed by SDS-PAGE and Western blotting with antisera against ATP synthase subunits Atpβ, Atp4, Atp5, Atp6, Su d, Su j, Su f, Atp9, and Oxa1 as indicated. (C) Mitochondria harboring expressed Oxa1 or Oxa1_{His} proteins were lysed in Triton X-100 containing buffer and were subjected to Ni-NTA purification. The purified material (indicated by B) and 10% of the unbound material (U) were subjected to SDS-PAGE, Western blotting, and immunodecoration with Oxa1 and Atp5 antisera as indicated. (D) Mitochondria harboring

expressed Oxa1_{His} (200 μg protein) or histidine-tagged Atpβ (Atpβ_{His}; 50 μg protein) were solubilized with buffer containing 1% Triton X-100 as indicated and incubated with Ni-NTA beads. The Ni-NTA-purified material was analyzed by SDS-PAGE and silver staining. (E) Mitochondria harboring Oxa1_{His} 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 as described in A. (F) In organello translation was performed in Oxa1_{His} containing mitochondria in the presence of CCCP (100 μM), oligomycin (10 μg/ml + oligo), valinomycin (0.5 μM, +Val), or ethanol (+EtOH) as a control, for 30 min at 25°C. After the addition of cold methionine, mitochondria were reisolated and subjected to SDS-PAGE and autoradiography. The position of the radiolabeled Atp9-Oxa1_{His} SDS-resistant complex is indicated. The asterisks (**) and (*) in A, C, and D indicate the positions of Oxa1_{His} and a C-terminal degradation product of Oxa1_{His}, respectively.

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 $\Delta oxa1$ 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 F₀-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 $\Delta oxa1$ 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_0 -sector, a step that involves its interaction with the Atp6 protein, is perturbed in the $\Delta oxa1$ mitochondria.

Oxa1 Copurifies with Components of the F_1F_0 -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_{His}) expressed in the $\Delta oxa1$ strain and performed affinity purification of the Oxa1_{His} and its associating proteins, using metal chelating chromatography. Mitochondria harboring the Oxa1_{His} derivative were lysed with Triton X-100, and the Oxa1_{His} protein was purified on Ni-NTA agarose beads. Oxa1_{His} and copurifying proteins were analyzed by SDS-PAGE followed by silver staining (Figure 5A). In addition to Oxa1_{His} (38 kDa), a number of other polypeptides were observed to copurify with Oxa1_{His}. We verified the identity of these Oxa1-associating proteins as subunits of the ATP synthase complex by either MALDI-TOF analysis (Atp α and Atp β subunits) or through Western blot analysis using subunit specific antisera (Atp α , Atp β , 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_{His}, because when mitochondrial lysis and purification was performed using the more stringent detergent SDS, only Oxa1_{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_{His} on the beads was complete (Figure 5C). Approximately 5% of the Atp5 protein was recovered with Oxa1_{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_{His} may represent an assembled F_1F_0 -complex. Analysis of the electrophoretic profile of the subunits, which copurified with Oxa1_{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 β -subunit of the F_1 -sector (Mueller *et al.*, 2004; Figure 5D), suggesting that an intact or very late assembly intermediate of the ATP synthase complex was associated with Oxa1.

The recovery of the ATP synthase components with the Oxa1_{His} was affected by the presence of the F_1F_0 -ATP synthase specific inhibitor, oligomycin. When mitochondria harboring the Oxa1_{His} derivative were incubated with oligomycin before lysis and Ni-NTA agarose chromatography, the ATP synthase subunits failed to copurify with the Oxa1_{His} protein (Figure 5E). The recovery of the Oxa1_{His} protein itself on the Ni-NTA beads was not affected by the oligomycin (Figure 5E). As mentioned earlier, oligomycin binds to a hydrophobic region of Atp9 that interfaces with Atp6 and by doing so blocks the transfer of H^+ ions between these proteins. Thus the binding of oligomycin may interfere with the ability of Atp9 to bind in a stable manner with the Oxa1 protein. 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*-chlorophenylhydrazine (CCCP) before detergent lysis and Ni-NTA 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_1F_0 -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_0 -sector-Oxa1 interface directly, rather than indirectly through an inhibition of the ATP synthesis activity of the F_1F_0 -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_0 -sector. It is therefore conceivable that in a given F_0 -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_{His} protein.

Association of Oxa1 with Newly Synthesized Atp9 Can Occur before It Assembles into an F_1F_0 -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_1F_0 -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_{His} with that associated with the F_1 -ATP synthase complex, using mitochondria harboring the Atp β _{His} derivative (Figure 6). Association of radiolabeled Atp9 with the Atp β _{His} protein would be observed if the in organello synthesized Atp9 protein can assemble with the F_1 -sector after synthesis in isolated mitochondria.

In organello translation in the presence of [³⁵S]methionine was performed in mitochondria bearing the Oxa1_{His} protein or Atp β _{His} protein and after translation, mitochondria were lysed in buffer containing Triton X-100 (detergent conditions that maintain the assembled F_1F_0 -ATP synthase complex intact) and the solubilized proteins were subjected to Ni-NTA chromatography. When translation was performed in the mitochondria bearing the Oxa1_{His} derivative, both the SDS-resistant Oxa1_{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

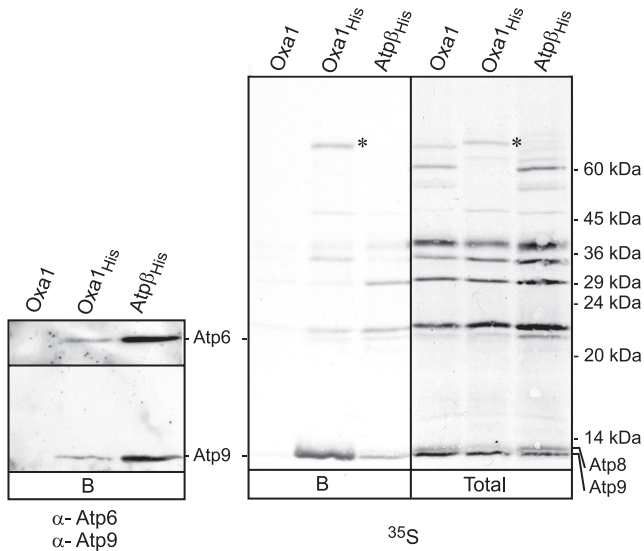


Figure 6. Oxa1 interacts with Atp9 before its assembly into an F_1F_0 -ATP synthase complex. In organello translation in the presence of [^{35}S]methionine was performed in mitochondria bearing overexpressed Oxa1 or Oxa1_{His} (Oxa1 or Oxa1_{His}) or in mitochondria harboring a histidine-tagged Atp β subunit (Atp β _{His}), as indicated. After a chase reaction as described in Figure 2, the mitochondria were reisolated and lysed in Triton X-100 containing buffer and subjected to Ni-NTA chromatography, as described in Figure 5D. The Ni-NTA bound material (indicated by B) and a sample corresponding to 10% of the total material applied to the Ni-NTA beads were analyzed by SDS-PAGE, Western blotting, and autoradiography (right panels, indicated by ^{35}S), followed by immunodecoration with Atp9 and Atp6 specific antisera (α -Atp9 and α -Atp6, respectively; left panel). The electrophoretic positions of Atp6, Atp8, and monomeric Atp9 are indicated. The position of the 72-kDa Oxa1-Atp9 SDS-resistant oligomer is indicated by an asterisk (*).

synthesized Atp9 found in association with the Atp β _{His} protein, however, was significantly lower than that observed together with the Oxa1_{His} protein (Figure 6, right panels), indicating that the majority of the radiolabeled Atp9 purified with Oxa1_{His} had not been assembled into an F_1F_0 -ATP synthase complex. On the other hand, immunodecoration with Atp9-specific antiserum showed that significantly more of the endogenous (i.e., pre-existing/assembled) Atp9 was recovered with the purified Atp β _{His} protein, than with the Oxa1_{His} 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 Atp β _{His} 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 Atp β _{His} and Oxa1_{His} comigrated with the immunodecorated 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. Finally, the recovery of both newly synthesized and pre-existing Atp9 on the Ni-NTA beads was specific for the presence of the histidine-tagged Oxa1 or Atp β derivatives, because no Atp9 was recovered on the beads when control mitochondria bearing nontagged versions of these proteins was analyzed in parallel (Figure 6).

Thus comparing the efficiencies of recovery of the radiolabeled versus endogenous Atp9 proteins with the Oxa1_{His} and Atp β _{His} proteins, we conclude that the majority of the radio-

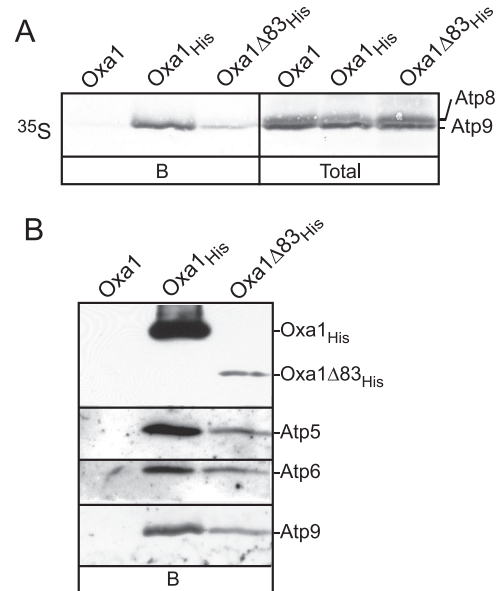


Figure 7. The C-terminal region of Oxa1 is not required for the Atp9-Oxa1 interaction. (A and B) Mitochondria were isolated from yeast strains expressing Oxa1, Oxa1_{His}, or Oxa1 Δ 83_{His} derivatives under control of the GAL10 promoter. (A) In organello translation in the presence of [^{35}S]methionine was performed in the mitochondria, 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 Oxa1_{His} and Oxa1 Δ 83_{His} on the Ni-NTA beads were detected using an Oxa1 antibody specific for the N-terminal region of Oxa1.

labeled Atp9 protein associated with Oxa1_{His} is not assembled into a F_1F_0 -ATP synthase complex. Rather, a significant fraction of the purified radiolabeled Atp9 protein represents an intermediate on the assembly pathway, which accumulates with Oxa1 after synthesis in isolated mitochondria.

Newly Synthesized Atp9 Can Associate with C-Terminally Truncated Oxa1

Finally, we investigated whether the C-terminal matrix located region of Oxa1 is required for the interaction of Oxa1 with newly synthesized Atp9 or with the endogenous ATP synthase subunits. In organello translation was performed in mitochondria carrying expressed C-terminally His-tagged 83 amino acids truncated Oxa1 (Oxa1 Δ 83_{His}). After translation, the mitochondria were solubilized by Triton X-100 containing buffer and subjected to Ni-NTA affinity purification (Figure 7). Both newly synthesized and endogenous Atp9 could be purified on the Ni-NTA beads together with Oxa1 Δ 83_{His} (Figure 7, A and B). In addition, immunodecoration with Atp5 and Atp6 antisera indicated the ability of other ATP synthase subunits to copurify with the Oxa1 Δ 83_{His} protein. Thus, as was observed with the full-length Oxa1_{His} derivative, the ATP synthase can be purified together with the C-terminally truncated Oxa1 Δ 83_{His} derivative. The efficiency of purification of ATP synthase subunits appeared to be lower with the Oxa1 Δ 83_{His} derivative than with the full-length Oxa1_{His} protein. However, immunodecoration of the Ni-NTA purified material with an N-terminal specific Oxa1 antiserum indicated that the levels of Oxa1 Δ 83_{His} recovered on the beads was drastically lower than that of the full-length Oxa1_{His} protein (Figure 7B). Immunodecoration of total mitochondrial protein with the N-terminal Oxa1 antiserum indicated that the overall steady state levels of Oxa1 Δ 83_{His} were

strongly reduced relative to Oxa1_{His} and this accounts for the reduced level of Oxa1Δ83_{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 Oxa1Δ83_{His} derivative reflects the lowered levels of Oxa1Δ83_{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 Oxa1Δ83_{His} derivative, because previous studies have shown that the levels of the Oxa1Δ83 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 F₁F_o-ATP synthase complex in yeast mitochondria. In the absence of Oxa1, a strong decrease in the levels of F₁F_o-ATPase enzyme activity was observed, and this decrease corresponds to a reduction in the overall protein levels of the assembled F₁F_o-ATP synthase complex. The severely reduced levels of F₁F_o-ATP synthase complex observed in the Δ*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 Δ*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 mitochondrially 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 F₁F_o-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_{out}-C_{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_{out}-C_{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 mitochondrially encoded proteins.

We demonstrate here that Oxa1 can directly associate with the newly synthesized Atp9 protein before its assembly into an intact F₁F_o-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_o-sector.

Consistently, the results presented here demonstrate the ability of Atp9 to assemble into its oligomeric form and to associate with F₁-sector subunits in the absence of Oxa1. Indeed the size exclusion chromatography experiments demonstrate that the Atp9-F₁-subcomplex present in the Δ*oxa1* mitochondria is similar to those in the Δ*atp10* strain, a mutant reported to be defective in the step of integration of the Atp6 protein into late Atp9-F₁-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 mitochondrially encoded Atp9 subunit. The involvement of Oxa1 in the assembly of the mitochondrial F₁F_o-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-F₁ 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-F₁-subcomplex represents the step that is adversely affected in the Δ*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 F₁F_o-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 F_0 -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 coassembly 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 F_1F_0 -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 F_1F_0 -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 F_0 -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 Oxa1_{His} was very similar to that affinity purified directly using a His-tagged Atp β 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 F_0 -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 Oxa1_{His} 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 F_1F_0 -subunits was also observed when the Oxa1_{His} 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 Oxa1_{His} on the Ni-NTA beads, but these levels paralleled the lower content of the Oxa1_{His} 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 F_1F_0 -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 F_1F_0 -ATP synthase com-

plex can be found with the Mdl1 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 Mdl1 with the Oxa1_{His} 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 F_0 -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 F_1F_0 -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 F_1F_0 -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 F_1F_0 -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.

ACKNOWLEDGMENTS

We thank Dr. Jean Velours (Bordeaux) for the generous gift of the Atp4 and Atp9 antibodies and Dr. Rod Devenish (Monash) for the Atp5 and Su d subunit antibodies. We are grateful to Dr. David Mueller (The Chicago Medical School, Chicago, IL) for giving us the yeast strain expressing the histidine-tagged Atp β protein and Dr. Sharon Ackerman (Wayne State School of Medicine, Detroit, MI) for the kind gift of the Δ *atp10* strain. This research was supported by funding from the National Science Foundation Grant M.C.B. 0347025 and U.S. Public Health Service Grant R01GM61573 to R.A.S.

REFERENCES

- Ackerman, S. H., and Tzagoloff, A. (2005). Function, structure, and biogenesis of mitochondrial ATP synthase. *Prog. Nucleic Acid Res. Mol. Biol.* 80, 95–133.
- Altamura, N., Capitanio, N., Bonnefoy, N., Papa, S., and Dujardin, G. (1996). The *Saccharomyces cerevisiae* OXA1 gene is required for the correct assembly of cytochrome c oxidase and oligomycin-sensitive ATP synthase. *FEBS Lett.* 382, 111–115.
- Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., and Schagger, H. (1998). Yeast mitochondrial F_1F_0 -ATP synthase exists as a dimer: identification of three dimer-specific subunits. *EMBO J.* 17, 7170–7178.
- Bauer, M., Behrens, M., Esser, K., Michaelis, G., and Pratje, E. (1994). PET1402, a nuclear gene required for proteolytic processing of cytochrome oxidase subunit 2 in yeast. *Mol. Gen. Genet.* 245, 272–278.
- Bonnefoy, N., Chalvet, F., Hamel, P., Slonimski, P. P., and Dujardin, G. (1994). OXA1, a *Saccharomyces cerevisiae* nuclear gene whose sequence is conserved from prokaryotes to eukaryotes controls cytochrome oxidase biogenesis. *J. Mol. Biol.* 239, 201–212.

- Capaldi, R. A., and Aggeler, R. (2002). Mechanism of the F_1F_0 -type ATP synthase, a biological rotary motor. *Trends Biochem. Sci.* 27, 154–160.
- Celebi, N., Yi, L., Facey, S. J., Kuhn, A., and Dalbey, R. E. (2006). Membrane biogenesis of subunit II of cytochrome *bo* oxidase: contrasting requirements for insertion of N-terminal and C-terminal domains. *J. Mol. Biol.* 357, 1428–1436.
- Cruciat, C., Brunner, S., Baumann, F., Neupert, W., and Stuart, R. A. (2000). The cytochrome *bc*₁ and cytochrome *c* oxidase complexes associate to form a single supracomplex in yeast mitochondria. *J. Biol. Chem.* 275, 18093–18098.
- Devenish, R. J., Prescott, M., Roucou, X., and Nagley, P. (2000). Insights into ATP synthase assembly and function through the molecular genetic manipulation of subunits of the yeast mitochondrial enzyme complex. *Biochim. Biophys. Acta* 1458, 428–442.
- du Plessis, D. J., Nouwen, N., and Driessen, A. J. (2006). Subunit a of cytochrome *o* oxidase requires both YidC and SecYEG for membrane insertion. *J. Biol. Chem.*, 281, 12248–12252.
- Funes, S., Nargang, F. E., Neupert, W., and Herrmann, J. M. (2004). The Oxa2 protein of *Neurospora crassa* plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family. *Mol. Biol. Cell* 15, 1853–1861.
- Galluhn, D., and Langer, T. (2004). Reversible assembly of the ATP-binding cassette transporter Mdl1 with the F_1F_0 -ATP synthase in mitochondria. *J. Biol. Chem.* 279, 38338–38345.
- Gerdes, L., Bals, T., Klostermann, E., Karl, M., Philipp, K., Hunken, M., Soll, J., and Schunemann, D. (2006). A second thylakoid membrane-localized Alb3/Oxa1/YidC homologue is involved in proper chloroplast biogenesis in *Arabidopsis thaliana*. *J. Biol. Chem.* 281, 16632–16642.
- Hell, K., Herrmann, J., Pratje, E., Neupert, W., and Stuart, R. A. (1997). Oxa1p mediates the export of the N- and C-termini of pCoxII from the mitochondrial matrix to the intermembrane space. *FEBS Lett.* 418, 367–370.
- Hell, K., Herrmann, J. M., Pratje, E., Neupert, W., and Stuart, R. A. (1998). Oxa1p, an essential component of the N-tail protein export machinery in mitochondria. *Proc. Natl. Acad. Sci. USA* 95, 2250–2255.
- Hell, K., Neupert, W., and Stuart, R. A. (2001). Oxa1p acts as a general membrane insertion machinery for proteins encoded by mitochondrial DNA. *EMBO J.* 20, 1281–1288.
- Herrmann, J. M., Stuart, R. A., Craig, E. A., and Neupert, W. (1994). Mitochondrial heat shock protein 70, a molecular chaperone for proteins encoded by mitochondrial DNA. *J. Cell Biol.* 127, 893–902.
- Jia, L., Dienhart, M., Schramm, M., McCauley, M., Hell, K., and Stuart, R. A. (2003). Yeast Oxa1 interacts with mitochondrial ribosomes: the importance of the C-terminal region of Oxa1. *EMBO J.* 22, 6438–6447.
- John, U. P., and Nagley, P. (1986). Amino acid substitutions in mitochondrial ATPase subunit 6 of *Saccharomyces cerevisiae* leading to oligomycin resistance. *FEBS Lett.* 207, 79–83.
- Kol, S., Turrell, B. R., de Keyser, J., van der Laan, M., Nouwen, N., and Driessen, A. J. (2006). YidC-mediated membrane insertion of assembly mutants of subunit c of the F_1F_0 ATPase. *J. Biol. Chem.* 281, 29762–29768.
- Kuhn, A., Stuart, R., Henry, R., and Dalbey, R. E. (2003). The Alb3/Oxa1/YidC protein family: membrane-localized chaperones facilitating membrane protein insertion? *Trends Cell Biol.* 13, 510–516.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lemaire, C., Guibet-Grandmougin, F., Angles, D., Dujardin, G., and Bonnefoy, N. (2004). A yeast mitochondrial membrane methyltransferase-like protein can compensate for oxa1 mutations. *J. Biol. Chem.* 279, 47464–47472.
- Moore, M., Harrison, S., Peterson, E. C., and Henry, R. (2000). Chloroplast Oxa1p homolog albino3 is required for post-translational integration of the light harvesting chlorophyll-binding protein into thylakoid membranes. *J. Biol. Chem.* 275, 1529–1532.
- Mueller, D. M., Puri, N., Kabaleeswaran, V., Terry, C., Leslie, A. G., and Walker, J. E. (2004). Ni-chelate-affinity purification and crystallization of the yeast mitochondrial F_1 -ATPase. *Protein Expr. Purif.* 37, 479–485.
- Nagley, P., Hall, R. M., and Ooi, B. G. (1986). Amino acid substitutions in mitochondrial ATPase subunit 9 of *Saccharomyces cerevisiae* leading to oligomycin or venturicidin resistance. *FEBS Lett.* 195, 159–163.
- Nunnari, J., Fox, T. D., and Walter, P. (1993). A mitochondrial protease with two catalytic subunits of nonoverlapping specificities. *Science* 262, 1997–2004.
- Preuss, M., Ott, M., Funes, S., Lührink, J., and Herrmann, J. M. (2005). Evolution of mitochondrial Oxa proteins from bacterial YidC. Inherited and acquired functions of a conserved protein insertion machinery. *J. Biol. Chem.* 280, 13004–13011.
- Saddar, S., and Stuart, R. A. (2005). The yeast F_1F_0 -ATP synthase: analysis of the molecular organization of subunit g and the importance of a conserved GXXXG motif. *J. Biol. Chem.* 280, 24435–24442.
- Saracco, S. A., and Fox, T. D. (2002). Cox18p is required for export of the mitochondrially encoded *Saccharomyces cerevisiae* Cox2p C-tail and interacts with Pnt1p and Mss2p in the inner membrane. *Mol. Biol. Cell* 13, 1122–1131.
- Souza, R. L., Green-Willms, N. S., Fox, T. D., Tzagoloff, A., and Nobrega, F. G. (2000). Cloning and characterization of COX18, a *Saccharomyces cerevisiae* PET gene required for the assembly of cytochrome oxidase. *J. Biol. Chem.* 275, 14898–14902.
- Stock, D., Leslie, A. G. W., and Walker, J. E. (1999). Molecular architecture of the rotary motor in ATP synthase. *Science* 286, 1700–1705.
- Stuart, R. A. (2002). Insertion of proteins into the inner membrane of mitochondria: the role of the Oxa1 complex. *Biochem. Biophys. Acta* 1592, 79–87.
- Sundberg, E., Slagter, J. G., Fridborg, I., Cleary, S. P., Robinson, C., and Coupland, G. (1997). ALBINO3, an *Arabidopsis* nuclear gene essential for chloroplast differentiation, encodes a chloroplast protein that shows homology to proteins present in bacterial membranes and yeast mitochondria. *Plant Cell* 9, 717–730.
- Szyrach, G., Ott, M., Bonnefoy, N., Neupert, W., and Herrmann, J. M. (2003). Ribosome binding to the Oxa1 complex facilitates co-translational protein insertion in mitochondria. *EMBO J.* 22, 6448–6457.
- Tzagoloff, A., Barrientos, A., Neupert, W., and Herrmann, J. M. (2004). Atp10p assists assembly of Atp6p into the F_0 unit of the yeast mitochondrial ATPase. *J. Biol. Chem.* 279, 19775–19780.
- van Bloois, E., Haan, G. J., de Gier, J. W., Oudega, B., and Lührink, J. (2004). F_1F_0 ATP synthase subunit c is targeted by the SRP to YidC in the *E. coli* inner membrane. *FEBS Lett.* 576, 97–100.
- van Bloois, E., Haan, G. J., de Gier, J. W., Oudega, B., and Lührink, J. (2006). Distinct requirements for translocation of the N-tail and C-tail of the *Escherichia coli* inner membrane protein CyoA. *J. Biol. Chem.* 281, 10002–10009.
- van der Laan, M., Bechtluft, P., Kol, S., Nouwen, N., and Driessen, A. J. (2004). F_1F_0 ATP synthase subunit c is a substrate of the novel YidC pathway for membrane protein biogenesis. *J. Cell Biol.* 165, 213–222.
- van der Laan, M., Nouwen, N. P., and Driessen, A. J. (2005). YidC—an evolutionary conserved device for the assembly of energy-transducing membrane protein complexes. *Curr. Opin. Microbiol.* 8, 182–187.
- Velours, J., and Arselin, G. (2000). The *Saccharomyces cerevisiae* ATP synthase. *J. Bioenerg. Biomembr.* 32, 383–390.
- Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 10, 1793–1808.
- Wittig, I., and Schägger, H. (2005). Advantages and limitations of clear-native PAGE. *Proteomics* 5, 4338–4346.
- Yen, M. R., Harley, K. T., Tseng, Y. H., and Saier, M. H., Jr. (2001). Phylogenetic and structural analyses of the oxa1 family of protein translocases. *FEMS Microbiol. Lett.* 204, 223–231.