The F₁F₀-ATP Synthase Complex Influences the Assembly State of the Cytochrome bc₁-Cytochrome Oxidase Supercomplex and Its Association with the TIM23 Machinery*

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The enzyme complexes involved in mitochondrial oxidative phosphorylation are organized into higher ordered assemblies termed supercomplexes. Subunits e and g (Su e and Su g, respectively) are catalytically nonessential subunits of the F₁F₀-ATP synthase whose presence is required to directly support the stable dimerization of the ATP synthase complex. We report here that Su g and Su e are also important for securing the correct organizational state of the cytochrome bc₁-cytochrome oxidase (COX) supercomplex. Mitochondria isolated from the Δsu e and Δsu g null mutant strains exhibit decreased levels of COX enzyme activity but appear to have normal COX subunit protein levels. An altered stoichiometry of the cytochrome bc₁-COX supercomplex was observed in mitochondria deficient in Su e and/or Su g, and a perturbation in the association of Cox4, a catalytically important subunit of the COX complex, was also detected. In addition, an increase in the level of the TIM23 translocase associated with the cytochrome bc₁-COX supercomplex is observed in the absence of Su e and Su g. Together, our data highlight that a further level of complexity exists between the oxidative phosphorylation supercomplexes, whereby the organizational state of one complex, i.e. the ATP synthase, may influence that of another supercomplex, namely the cytochrome bc₁-COX complex.

ATP is produced within mitochondria through the oxygen-consuming process termed oxidative phosphorylation (OXPHOS). The protein complexes involved in the mitochondrial OXPHOS pathway are large multisubunit enzymes, commonly referred to as complexes I–V. Complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome bc₁-complex), complex IV (cytochrome c oxidase, COX) compose the electron transport chain complexes and the F₁F₀-ATP synthase complex, which directly synthesizes ATP and is often referred to as complex V (1–3). Although complexes I–V can be detergent-solubilized and purified from the mitochondrial membranes as independent enzymatically active complexes, recent findings indicate that these complexes do not exist as physically separate entities within the mitochondrial inner membrane. Rather subpopulations of OXPHOS complexes physically associate with each other to form larger assemblies termed “OXPHOS supercomplexes” (4–17). Evidence for the formation of complex III–IV supercomplex (termed here the cytochrome bc₁-COX supercomplex) and complex I–III–IV supercomplex, as well as dimeric/oligomeric F₁F₀-ATP synthase complexes, has been reported (4–17).

Formation of OXPHOS supercomplexes in mitochondria has been conserved throughout evolution, and determination of their functional relevance represents a new developing area of mitochondrial biogenesis research. The supercomplex organizational state of the OXPHOS complexes may confer an enzymatic advantage to these complexes as it has been proposed to enable direct substrate channeling between the complexes (4, 5, 12, 17). In addition the supercomplex organizational state of the ATP synthase plays a role in establishing the architecture of the mitochondrial inner membrane, the principal site of the OXPHOS activity (18–27). Furthermore, a subset of the TIM23 translocase, involved in the import of nuclearly encoded proteins across the inner membrane, has been shown to exist in association with a subpopulation of the cytochrome bc₁-COX supercomplex (28). Finally, it has recently been demonstrated that a subpopulation of the cytochrome bc₁-COX supercomplex can be found in association with the assembly factors Cox4 and Shy1, which are involved in the biogenesis of the COX complex (29). Thus it appears that a heterogeneity exists within populations of the cytochrome bc₁-COX supercomplex and that different organizational states of these OXPHOS complexes may exist within one given mitochondrial type, thereby adding to the complexity of analyzing and understanding the role(s) of the OXPHOS supercomplex assembly states.

The F₁F₀-ATP synthase complexes exist as higher ordered structures that, under mild detergent conditions, can be solubilized from mitochondrial membranes as dimeric ATP synthase complexes. To date, the dimeric assembly state of the ATP synthase has been best characterized from the yeast Saccharomyces cerevisiae, where it has been proposed that the ATP synthase dimers are solubilized from a larger network of ATP synthase oligomers within the mitochondrial inner membrane (6, 7, 10, 18–24, 30–38). The F₁F₀-ATP synthase complex can be divided into two regions, the membrane-embedded,
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H+ -pumping F0 sector and the hydrophilic, catalytic F1 sector located in the mitochondrial matrix (2). Dimerization of the ATP synthase complex involves F0-F0 interactions and in a manner involves an interface formed by the F0 sector subunits e, g, and 4 (Su e, Su g, and Atp4, respectively). Su e and Su g proteins (in contrast to Atp4) are not essential for the enzymatic activity of the ATP synthase complex, but like Atp4, Su e and Su g are required to support the stable dimerization of the ATP synthase complex. Current evidence indicates that in the absence of Su e and/or Su g, the ATP synthase dimers can still form in the mitochondrial membrane but that these dimers are not stable under detergent solubilization conditions; hence, monomeric complexes are predominantly observed in extracts of Δsu e and Δsu g mitochondria (30–37). As indicated earlier, the dimeric/oligomeric state of the ATP synthase plays a role in the development of the cristae membrane architecture (18–27). Cristae membranes have been reported to be absent in the su e and su g null yeast mutants and instead the inner membrane has been shown to adopt a proliferated "onion-like" morphology (18–20).

A number of reports in the literature have implicated that the assembly and/or stability of the cytochrome oxidase (COX) complex may be related to that of the ATP synthase complex (39–42). As ATP synthase assembly mutants often exhibit instability in their mtDNA (and hence coding capacity for key COX subunits), it could be argued that the reduced COX activity could merely be due to partial loss of the mtDNA in these strains. Reduced COX enzyme activity, however, was also recently reported in atp6 mutants, where retention of the mtDNA had been ensured by growing the cells under appropriate selective pressure, thereby suggesting that loss of the ATP synthase complex and/or enzyme activity can negatively impact the COX complex in a direct manner (40, 41). Furthermore, the F0 sector subunit Su g has also been reported to be required for maximal COX enzyme activity despite the fact that spectral analysis indicated that Δsu g mitochondria display a normal cytochrome content (42). As Su g is not an essential subunit of the ATP synthase complex, this observation also suggests that the Su e/Su g proteins themselves, rather than the enzymatic activity of the ATP synthase complex, may directly impact the enzymatic activity of the COX complex.

In this study, we have further pursued the relationship between the ATP synthase dimer-specific subunits, Su g and Su e, and the COX complex. We demonstrate here that the presence of both Su g and Su e are directly required for the establishment of maximal COX enzyme activity. Furthermore, in the absence of Su g and Su e, we observed an alteration in the assembly state of the cytochrome bc1 complex, whereby the stoichiometry of the cytochrome bc1-COX complex was altered, and free cytochrome bc1 complexes were observed. In addition, an increased level of cytochrome bc1-COX complexes associated with the TIM23 translocase of the inner membrane was also observed in the absence of Su g and/or Su e. Taken together our findings indicate that the absence of Su g and Su e not only affects the dimeric state of the ATP synthase but also causes an imbalance in the higher ordered organizational states of other OXPHOS supercomplexes and their association with the TIM23 machinery.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—S. cerevisiae strains used in this study are wild type (WT) (W303-1A, Mata, leu2, trpl1, ura3, his3, ade2), Δsu g (W303-1A, ATP20::HIS3), Δsu e (W303-1A, TIM11::HIS3), Δcox4 (W303-1A, COX4::TRP1), Δcyt1 (W303-1A, CYT1::HIS3), Δimp1 (W303-1A, IMP1::HIS3), Δsdh2 (BY4739α, SDH2::KAN) and Δsdh4 (BY4739α, SDH4::KAN) (6, 8, 32). Yeast strains were maintained and cultured using standard protocols at 30 °C on YP-Gly (YP, 0.5% lactate media supplemented with 3% glycerol) (WT, Δsu g, and Δsu e) or YP-Gal (YP, 0.5% lactate media supplemented with 2% galactose) (Δcox 4, Δcyt1, Δimp1, Δsdh2 and Δsdh4) as indicated.

Rho0/rho− Cell Conversion Assay—Rho0/rho− assay was performed as described earlier (23). Briefly, yeast strains were grown on YP-lactate medium supplemented with 3% glycerol or 2% galactose overnight at 30 °C. The following day, equal numbers of cells were plated from each strain onto YPD (2% glucose) plates or YP-Gly plates containing 0.1% galactose. Following incubation at 30 °C, the colonies were counted, and the number of rho0− cells (i.e. petite cells) was calculated and expressed as a percentage of total cells.

Clear Native-Gel Electrophoresis—CN-PAGE analysis of the F0-F0-ATP synthase and cytochrome bc1-COX supercomplexes was performed essentially as described previously (23, 37). Mitochondria (200 μg of protein) were solubilized in lysis buffer (34 mM potassium acetate, 34 mM HEPES-KOH, pH 7.4, 11.4% glycerol, and 1 mM phenylmethylsulfonyl fluoride) with digitonin for 30 min on ice and then were subjected to a clarifying spin of 30,000 g for 30 min at 4 °C. The supernatant from each sample was analyzed on a 3.5–10% gradient gel, Western-blotted, and immunodecorated with subunit-specific antibodies as indicated.

Two-dimensional Blue Native-SDS-PAGE—Mitochondria were solubilized with 0.5% digitonin and analyzed by blue native (BN)-gel electrophoresis as described previously (6). Following BN-PAGE, the individual gel strips were excised, and SDS-PAGE were performed according to published methods.

Gel Filtration Analysis—Isolated mitochondria (1 mg of protein) were solubilized in 1% Triton X-100, 150 mM NaCl, 20 mM HEPES-KOH, pH 7.2, 2 mM phenylmethylsulfonyl fluoride buffer. Following a clarifying spin, the detergent extract was applied to a Superose 6 FPLC gel filtration column (25-mL column volume). Fractions (0.3 mL) were collected, precipitated with trichloroacetic acid, and analyzed by SDS-PAGE, Western blotting, and immunodecoration with Cox2- and Cox4-specific antisera.

Miscellaneous—Mitochondrial isolation, protein determinations, and SDS-PAGE were performed according to published methods.
enzyme activities were performed on freshly isolated mitochondria, as described previously (8).

**RESULTS**

The Dimeric ATP Synthase Is Required for Maximal Cytochrome Oxidase Activity—A reduction in COX enzyme activity has been reported in yeast mutants deficient in Su g of the ATP synthase (42). As yeast cells deficient in Su e and Su g are known to undergo a high frequency of spontaneous rho−/rho0 conversion (i.e. deletion and/or loss of mtDNA) when grown on fermentable carbon sources, the decrease in COX enzyme activity could simply have reflected the instability of the mtDNA in the ∆su g cells (23, 37). Loss of mtDNA would result in a reduced coding capacity for the COX subunits, 1–3, the presence of which are essential for the enzyme activity of the COX complex.

Growth of the ∆su e and ∆su g strains under nonfermentable carbon source (glycerol) conditions exerts a selective pressure on these strains to maintain the integrity of their mitochondrial genome, as demonstrated by the fact that ~97% of the resulting ∆su e and ∆su g cells were determined to be rho+, i.e. contained functional mtDNA (Fig. 1A). On the other hand, overnight growth of these null mutant strains in a fermentable carbon source (galactose), as published previously (23, 37), resulted in a significant instability of the mtDNA, as 45 and 50% of the ∆su e and ∆su g cells, respectively, had developed a rho9/rho− phenotype (Fig. 1A).

Mitochondria isolated from the ∆su g and ∆su e strains, which had been maintained in glycerol medium, were therefore analyzed for their COX and cytochrome bc1 enzyme activity levels. The level of the COX enzyme activity was affected in the absence of Su g or Su e, as a decrease of ~25 and 45% of the wild type activity levels were measured in the ∆su g and ∆su e mitochondria, respectively (Fig. 1B, left panel). On the other hand, the level of the cytochrome bc1 enzyme activity in the ∆su g and ∆su e mitochondria was in the range of that measured for the wild type control samples (Fig. 1B, right panel). We therefore conclude that maximal COX activity, but not cytochrome bc1 activity, requires the presence of subunits e and g of the ATP synthase complex. As cytochrome b is encoded by the mtDNA, the normal levels of cytochrome bc1 activity measured in the ∆su e and ∆su g mitochondria further support that the integrity of the mtDNA has been maintained in these strains when grown on glycerol-based media.

To investigate whether the observed reduction of COX enzyme activity in the ∆su g and ∆su e mitochondria reflected an overall decrease in the levels of the COX proteins, the steady
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state levels of individual COX subunits were analyzed by SDS-PAGE and immune blotting with subunit-specific antisera. The levels of the COX subunits tested (Cox2, Cox4, and Cox5α) in the Δsu e and Δsu g mitochondria were similar to those in the wild type control (Fig. 1C). As anticipated from the enzyme measurements, the steady state levels of the cytochrome bc₁ complex subunits (Core1, Rieske FeS, cytochrome c₁, and cytochrome b) were not significantly compromised in the absence of subunits e and g. Interestingly, an increase in the levels of cytochrome c₁, an intermembrane space localized protein that functions to deliver electrons from the cytochrome bc₁ complex to the cytochrome oxidase complex, was observed in both the Δsu e and Δsu g mitochondria, relative to the wild type control (Fig. 1D). The observed difference in levels of cytochrome c cannot be attributed to a difference in the intactness of the intermembrane space of the different mitochondrial preparations, as the levels of the soluble intermembrane space control proteins, cytochrome b₂ and cytochrome c peroxidase, in the Δsu g and Δsu e mitochondria were similar to those in the control wild type mitochondria (Fig. 1D).

In summary, we report here that the presence of Su g and Su e of the ATP synthase complex are required for optimal COX enzyme activity. The reduced levels of the COX enzyme activity in the absence of Su g and/or Su e appear not to be due to an overall decrease in the levels of COX protein subunits but rather reflect a change in the enzymatic property of the COX complex.

The Assembly State of the Cytochrome bc₁-COX Complex Is Altered in the Absence of Su g and Su e of the ATP Synthase—We have previously demonstrated that the cytochrome bc₁ complex physically associates with the COX complex to form the cytochrome bc₁-COX supercomplex (8, 9). To ascertain whether the assembly state of the cytochrome bc₁-COX supercomplex was affected in the absence of Su g and Su e, mitochondria from the Δsu g and Δsu e strains were solubilized with digitonin and analyzed using CN-PAGE, followed by Western blotting and immunedecoration with antiserum against Core1, a subunit of the cytochrome bc₁ complex (Fig. 2). As demonstrated previously in wild type mitochondria, when solubilized with the mild detergent, digitonin, the cytochrome bc₁-COX supercomplex, displays an apparent molecular mass of ~1000 kDa (Fig. 2). In the absence of subunit g (i.e. in Δsu g mitochondria), a perturbation in the organizational state of the cytochrome bc₁-COX supercomplex was observed, and the presence of smaller assembly forms of the cytochrome bc₁ complexes, whose sizes are in range of the dimeric (~670 kDa) and possibly monomeric cytochrome bc₁ complexes, was also detected (Fig. 2, upper panel). The cytochrome bc₁ supercomplexes present in the Δsu g mitochondria appeared to migrate as smaller complexes relative to that observed in the wild type control mitochondria. A similar perturbation in the assembly and stoichiometry of the cytochrome bc₁-COX supercomplex was also observed in the Δsu e mitochondria (Fig. 2, lower panel), suggesting that the presence of both Su e and Su g support the assembly of the cytochrome bc₁-COX supercomplex. Consistently, as was observed for the single mutant mitochondria, the assembly state of the cytochrome bc₁-COX supercomplex was significantly perturbed in mitochondria iso-

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**FIGURE 2.** Clear native gel analysis of the cytochrome bc₁-COX supercomplexes. Mitochondria were isolated from WT, Δsu g (upper panel), and Δsu e (lower panel) strains (all grown in YP-Gly medium) solubilized with digitonin (Dig), at concentrations indicated, and were subsequently analyzed on CN-PAGE, as described under “Experimental Procedures.” Following electrophoresis, the native gels were Western-blotted, and immunedecoration was performed using a yeast Core1-specific (α-Core1) antiserum. The positions of the cytochrome bc₁-COX supercomplex (III-IV) and of the complexes corresponding to the size of the dimeric (III) and monomeric (III) cytochrome bc₁ complex are indicated.

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lated from the double null mutant Δsu g, Δsu e, i.e. when both Su g and Su e are absent (results not shown).

Bearing in mind the protein levels of the cytochrome bc₁ and COX complex subunits were not significantly affected in the Δsu e and Δsu g mitochondria, these findings indicate that the
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**FIGURE 3.** Assembly of the cytochrome bc\(_1\)-COX complex and its association with the TIM23 machinery is altered in the absence of Su g of the ATP synthase. Mitochondria isolated from the WT (upper panel) and Δsu g (lower panel) strains were solubilized in digitonin (0.75%), clarified by centrifugation, and directly analyzed by BN-PAGE. After the separation of the solubilized complexes by BN-PAGE, proteins were further resolved by a two-dimensional SDS-PAGE step. Following Western blotting, immunodecoration with specific antisera against Core1, Cox2, Cox4, Atp4, Tim23, and Tim17 was performed as indicated. The positions of molecular weight markers are indicated by bars below the panel. Abbreviations are as in Fig. 2, and the position of the monomeric F\(_{1}\)F\(_{0}\)-ATP synthase complex is indicated by \(V_{\text{mono}}\).

ATP synthase subunits, Su g and Su e, influence the ability of the cytochrome bc\(_1\) and COX complexes to correctly assemble with normal stoichiometry and exist together in a stable supercomplex.

**The Absence of Su e and Su g May Affect the Stoichiometry and Stability of the Cytochrome bc\(_1\)-COX Supercomplexes**—To analyze the assembly states of the cytochrome bc\(_1\)-COX supercomplex in the absence of subunits Su e and Su g of the ATP synthase in more detail, we performed a BN-PAGE followed by a two-dimensional SDS-PAGE analysis (Fig. 3). The two-dimensional SDS-PAGE step following the native electrophoresis procedure serves to resolve the supercomplexes into their individual protein subunits and thereby enables the analysis of a number of separate components in parallel.

Immunodecoration of the SDS-PAGE-resolved complexes with antibodies specific for subunits of the cytochrome bc\(_1\) complex (Core1) and the COX complex (Cox2 and Cox4) confirmed that in wild type mitochondria the cytochrome bc\(_1\) complex was present almost exclusively in the COX-containing supercomplex form (indicated by III--IV*, Fig. 3, upper panel). Under these electrophoretic conditions, the wild type cytochrome bc\(_1\)-COX supercomplex closely migrated with the dimeric ATP synthase, as indicated by immunodecoration for Atp4, a subunit of the F\(_{0}\)-ATP synthase sector. Consistent with the data in Fig. 2, a perturbation in the assembly state of the cytochrome bc\(_1\)-COX complex was observed in the Δsu g mitochondria, with a significant fraction of the Core1 protein being detected in protein complex(es) whose sizes correspond to 600 kDa and smaller (Fig. 3, lower panel). Furthermore, the cytochrome bc\(_1\)-COX supercomplex that was present in the Δsu g mitochondria (indicated by III--IV*), exhibited a faster migration behavior than the supercomplex in the wild type control, suggesting that the stoichiometry of the cytochrome bc\(_1\)-COX arrangement may be altered in the absence of Su g. As published previously (6, 23, 37, 38), the ATP synthase complex in Δsu g mitochondria was predominantly solubilized as a monomeric complex (monitored by decoration for Atp4) (Fig. 3, lower panel).

In the Δsu g mitochondria, the Cox2 protein was recovered exclusively in the III--IV* form of the cytochrome bc\(_1\)-COX supercomplex, and no evidence of smaller Cox2-containing complexes were obtained indicating that assembly intermediates of the COX complex are not present in these mitochondria. On the other hand, the behavior of the peripheral COX subunit, Cox4, was markedly different in the Δsu g mitochondria relative to the wild type control (Fig. 3). A significant fraction of the Cox4 protein was found in lower molecular mass forms in the Δsu g mitochondria, as indicated by Cox4* and Cox4** in Fig. 3 (lower panel). In the control wild type mitochondria, the Cox4 protein remained firmly associated with the cytochrome bc\(_1\)-COX supercomplex, where its behavior mirrored that of the Cox2 protein. A similar result with regard to the organizational state of the cytochrome bc\(_1\) and COX complexes and the assembly state of the Cox4 protein was also observed in the Δsu e mitochondria (results not shown).

To further address whether any assembly intermediates of the COX complex existed in the Δsu g mitochondria, we analyzed the assembly state of the COX complex independent from its association with cytochrome bc\(_1\) complex. To do so, mitochondria were solubilized with Triton X-100, conditions that disrupt the association of the COX complex from the cytochrome bc\(_1\) complex, but maintain the integrity of the assembled COX complex. In wild type mitochondria, the assembled COX complex, as evidenced by co-fractionation of the Cox2 and Cox4 proteins, eluted from a gel filtration column with an apparent mass of 450–500 kDa (Fig. 4, upper panel). The COX complex from the Δsu g mitochondria (Fig. 4, lower panel) displayed very similar behavior, and no evidence of assembly intermediates containing Cox2, a key core subunit of the COX com-
plex, could be observed. As reported above, a fraction of the Cox4 protein was observed not to remain with the assembled COX complex in the Δsu:g mitochondria, but rather was fractionated in a low molecular mass fraction of ~30–40 kDa.

Taken together with the native gel electrophoresis results, we conclude that the COX complex can assemble in the absence of Su:g or Su:e, but the organizational state of the cytochrome bc₁-COX supercomplex is altered. A smaller assembly form of this supercomplex and free cytochrome bc₁ complexes appear to be the predominant forms of these OXPHOS complexes in the Δsu:e and Δsu:g mitochondria. Furthermore, in the absence of Su:g, the molecular environment of the Cox4 protein appears to be altered, such that it does not remain so firmly associated with the assembled COX complex, as in wild type control mitochondria.

An Increased Level of the TIM23 Complex Associated with the OXPHOS Supercomplexes Is Observed in the Absence of Su:g and Su:e—A subset of the cytochrome bc₁-COX supercomplexes have been shown to physically associate with a subpopulation of the TIM23 translocase (28). We therefore next addressed whether the fraction of the cytochrome bc₁-COX supercomplexes associated with the TIM23 was adversely affected by the absence of Su:g or Su:e.

The assembly state of the TIM23 translocases in digitonin extracts from wild type and Δsu:g mitochondria was monitored by BN-PAGE/two-dimensional SDS-PAGE, followed by immunodecoration for the Tim23 and Tim17 proteins, two key core subunits of the TIM23 complex (Fig. 3). As published previously, in wild type control the majority of the solubilized Tim23 and Tim17 proteins were present in a complex of ~150 kDa in mass, which most likely corresponds to the TIM23 complex. A smaller fraction was present in a larger TIM23 complex (of ~200 kDa), as indicated by TIM23* (46–49). In addition a small fraction of the Tim23 and Tim17 proteins was observed to co-migrate in a complex in the range of 1000 kDa, and this represents that fraction of the TIM23 complex that exists in association with the cytochrome bc₁-COX supercomplex (Fig. 3, upper panel). An alteration in the assembly state of the TIM23 complexes was observed in Δsu:g mitochondria, whereby an increase in the level of Tim23 and Tim17 components associated with the cytochrome bc₁-COX supercomplex was seen. Furthermore, in the absence of Su:g, an increase in the level of TIM23* complex, relative to the smaller TIM23 complex, was observed (Fig. 3, lower panel). Interestingly, this alteration in the ratio of TIM23*:TIM23 complexes observed in the Δsu:g mitochondria was more apparent for the Tim23 protein than for the Tim17 subunit. A similar increase in the ratio of TIM23*:TIM23 complexes and in the level of TIM23 associated with the cytochrome bc₁-COX supercomplex was also observed in the Δsu:e mitochondria (results not shown).

In summary, despite the reduced levels of the cytochrome bc₁-COX supercomplex present in Δsu:g mitochondria, an elevated level of cytochrome bc₁-COX-TIM23 complexes are formed. We therefore conclude that the absence of the stable dimeric state of the ATP synthase and/or the Su:e/Su:g proteins themselves affects the higher ordered assembly state of both the cytochrome bc₁-COX and the TIM23 complexes.

The Assembly State of the Cytochrome bc₁-COX Supercomplex Is Not Influenced by the Level of OXPHOS Activity in the Mitochondria—Although Δsu:e and Δsu:g strains exhibit only a slight growth defect on nonfermentable carbon sources, their absence has been suggested to partially compromise the mitochondrial inner membrane potential (24), a phenotype to which the decrease in COX activity is most likely contributing. Therefore, it could be argued that the observed perturbation of the assembly state of the cytochrome bc₁-COX supercomplex may be as a result of the reduced level of membrane potential in mitochondria deficient in Su:e or Su:g. To this end, the relationship between the assembly state of the cytochrome bc₁-COX supercomplex and the perturbation of the membrane potential was directly tested, using a variety of mitochondria isolated from cells that are known to have a severely compromised mitochondrial OXPHOS activity (Fig. 5).

Mitochondria isolated from the respiratory-deficient Δsdh2 and Δsdh4 yeast strains and the assembly state of the cytochrome bc₁-COX complex was analyzed by CN-PAGE following digitonin solubilization. SDH2 and SDH4 encode essential subunits of complex II of the electron transport chain (the succinate dehydrogenase complex). Assembly of an active complex II enzyme is lacking in the Δsdh2 and Δsdh4 mitochondria, resulting in an inhibition of OXPHOS activity and, accordingly, the inability of the sdh mutant strains to grow on nonfermentable carbon sources (50). CN-PAGE analysis indicated however that the assembly state of both the cytochrome bc₁-COX supercomplex and the dimeric ATP synthase enzyme was unaffected in the Δsdh2 or Δsdh4 mutant mitochondria (Fig. 5A). These results demonstrate that a reduced OXPHOS capacity does not affect the ability of the cytochrome bc₁-COX complex to assemble into its supercomplex form, and furthermore, the assembly of this supercomplex and also of the dimeric ATP synthase is independent of the assembly of an active complex II enzyme.

To further support that reduced OXPHOS activity is not the cause for perturbation on the cytochrome bc₁-COX supercomplex assembly observed in the Δsu:g and Δsu:e mitochondria, we analyzed the assembly state of the supercomplex in mitochondria isolated from cells grown in the presence of OXPHOS inhibitors, antimycin A (specific inhibitor of cytochrome bc₁ complex) and oligomycin (inhibits flux of H⁺ through F₁F₀-ATP synthase complex) (Fig. 5B). The assembly of both the cytochrome bc₁-COX supercomplex and the dimeric ATP synthase was found to be unaffected in mitochondria grown in the presence of these OXPHOS inhibitors (Fig. 5B).

Finally, we analyzed the assembly state of the cytochrome bc₁-COX supercomplex in mitochondria isolated from the cardiolipin-deficient strain, Δcrd1. CRD1 encodes the mitochondrial encoded cardiolipin synthase enzyme, and Δcrd1 mutant mitochondria have been reported to have a severely compromised membrane potential (9). Despite this reduced membrane potential, mitochondria isolated from the Δcrd1 mutant displayed a normal assembly state of the cytochrome bc₁-COX supercomplex and the dimeric ATP synthase (Fig. 5C). It is important to note that the cytochrome bc₁-COX complex has been shown to exhibit an inherent instability in the absence of cardiolipin, which causes it to fall apart under harsh conditions.
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The intact nature of the cytochrome bc\textsubscript{1}-COX complex from the Δcrd1 mutant thus demonstrates the mild nature of the solubilization/CN-PAGE conditions used here. We therefore propose that the observed perturbation of the cytochrome bc\textsubscript{1}-COX complex in the Δsu\textsubscript{g} or Δsu\textsubscript{e} mitochondria differs from that observed in the Δcrd1 mitochondria and thus may reflect an assembly, rather than an instability problem, caused by the absence of Su\textsubscript{g} or Su\textsubscript{e}. Taken together, these findings indicate that the perturbation of the cytochrome bc\textsubscript{1}-COX supercomplex assembly observed in the Δsu\textsubscript{g} and Δsu\textsubscript{e} mitochondria is not because of a possible indirect effect of the reduced energetic status of the mitochondria but rather is directly because of the physical absence of the Su\textsubscript{g} and Su\textsubscript{e} proteins.

The Assembled Cytochrome bc\textsubscript{1}-COX Supercomplex Is Not Required for Formation of ATP Synthase Dimers—The data thus far indicate that the subunits Su\textsubscript{e} and Su\textsubscript{g} of the ATP synthase complex are required to support both the assembly of ATP synthase dimers and also the correct assembly of the cytochrome bc\textsubscript{1}-COX complex. We addressed whether perturbations in the assembly of either the cytochrome bc\textsubscript{1}, or cytochrome oxidase complexes exerted an adverse effect on the Su\textsubscript{g} and Su\textsubscript{e} proteins or on their ability to support stable ATP synthase dimer formation. Mitochondria were isolated from the following cytochrome bc\textsubscript{1}- or COX-deficient yeast strains: Δcyt1 (deficient in cytochrome c\textsubscript{1} of the cytochrome bc\textsubscript{1} complex), Δcox4 (deficient in the essential Cox4 subunit of the COX complex), and Δimp1 strains (deficient in Imp1, required for maturation of Cox2 subunit, and hence are deficient in assembled COX complex). The assembled cytochrome bc\textsubscript{1}-COX supercomplex is absent in these strains because of the lack of an assembled cytochrome bc\textsubscript{1} or COX complex, respectively (8, 9). Neither the steady state levels of Su\textsubscript{g} and Su\textsubscript{e} (Fig. 6A) nor their ability to support the stable ATP synthase dimerization of F\textsubscript{1}F\textsubscript{0}-ATP synthase complexes were, however, adversely affected by the absence of an assembled cytochrome bc\textsubscript{1}-COX supercomplex in these strains (Fig. 6B). We therefore conclude that the assembly state of the cytochrome bc\textsubscript{1}-COX supercomplex does not affect that of the dimeric ATP synthase, despite the fact that the ATP synthase subunits Su\textsubscript{g} and Su\textsubscript{e} strongly influence the higher ordered assembly states of the cytochrome bc\textsubscript{1}-COX supercomplex.

**DISCUSSION**

To further investigate the proposed relationship between the ATP synthase and the COX complex, we investigated the native electrophoresis conditions, e.g. blue native-PAGE (9, 51). The intact nature of the cytochrome bc\textsubscript{1}-COX complex from the Δcrd1 mutant thus demonstrates the mild nature of the solubilization/CN-PAGE conditions used here. We therefore propose that the observed perturbation of the cytochrome bc\textsubscript{1}-COX complex in the Δsu\textsubscript{g} or Δsu\textsubscript{e} mitochondria differs from that observed in the Δcrd1 mitochondria and thus may reflect an assembly, rather than an instability problem, caused by the absence of Su\textsubscript{g} or Su\textsubscript{e}. Taken together, these findings indicate that the perturbation of the cytochrome bc\textsubscript{1}-COX supercomplex assembly observed in the Δsu\textsubscript{g} and Δsu\textsubscript{e} mitochondria is not because of a possible indirect effect of the reduced energetic status of the mitochondria but rather is directly because of the physical absence of the Su\textsubscript{g} and Su\textsubscript{e} proteins.

The Assembled Cytochrome bc\textsubscript{1}-COX Supercomplex Is Not Required for Formation of ATP Synthase Dimers—The data thus far indicate that the subunits Su\textsubscript{e} and Su\textsubscript{g} of the ATP synthase complex are required to support both the assembly of ATP synthase dimers and also the correct assembly of the cytochrome bc\textsubscript{1}-COX complex. We addressed whether perturbations in the assembly of either the cytochrome bc\textsubscript{1}, or cytochrome oxidase complexes exerted an adverse effect on the Su\textsubscript{g} and Su\textsubscript{e} proteins or on their ability to support stable ATP synthase dimer formation. Mitochondria were isolated from the following cytochrome bc\textsubscript{1}- or COX-deficient yeast strains: Δcyt1 (deficient in cytochrome c\textsubscript{1} of the cytochrome bc\textsubscript{1} complex), Δcox4 (deficient in the essential Cox4 subunit of the COX complex), and Δimp1 strains (deficient in Imp1, required for maturation of Cox2 subunit, and hence are deficient in assembled COX complex). The assembled cytochrome bc\textsubscript{1}-COX supercomplex is absent in these strains because of the lack of an assembled cytochrome bc\textsubscript{1} or COX complex, respectively (8, 9). Neither the steady state levels of Su\textsubscript{g} and Su\textsubscript{e} (Fig. 6A) nor their ability to support the stable ATP synthase dimerization of F\textsubscript{1}F\textsubscript{0}-ATP synthase complexes were, however, adversely affected by the absence of an assembled cytochrome bc\textsubscript{1}-COX supercomplex in these strains (Fig. 6B). We therefore conclude that the assembly state of the cytochrome bc\textsubscript{1}-COX supercomplex does not affect that of the dimeric ATP synthase, despite the fact that the ATP synthase subunits Su\textsubscript{g} and Su\textsubscript{e} strongly influence the higher ordered assembly states of the cytochrome bc\textsubscript{1}-COX supercomplex.

**DISCUSSION**

To further investigate the proposed relationship between the ATP synthase and the COX complex, we investigated the

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**FIGURE 5. Assembly of the cytochrome bc\textsubscript{1}-COX supercomplex or the dimeric ATP synthase is not adversely affected in membrane potentially compromised mitochondria.** A, mitochondria isolated from WT, Δsdh2, and Δsdh4 strains grown in YP-Gal medium were solubilized with digitonin (dig), at concentrations indicated, and analyzed by CN-PAGE, as described under "Experimental Procedures." Following electrophoresis, the native gels were Western-blotted, and immunodecoration was performed using either a Core1-specific antiserum to visualize the cytochrome bc\textsubscript{1}-COX supercomplex or a yeast F\textsubscript{1}-specific antiserum to analyze the assembly state of the ATP synthase. B, mitochondria isolated from WT and Δsdh1 strains grown in YP-Gal medium and supplemented with antimycin A (+AA) (1.5 μg/ml) or oligomycin (+Oli) (3 μg/ml) for 2 h prior to harvesting the cells for CN-PAGE analysis as described in A. Abbreviations are same as in Fig. 2, and the positions of the dimer (V\textsubscript{dim}) and monomeric (V\textsubscript{mon}) F\textsubscript{1}F\textsubscript{0}-ATP synthase complexes are indicated.
ATP Synthase Influences the Cytochrome bc$_1$-COX Complex

FIGURE 6. Assembly of the dimeric ATP synthase does not require the assembly of the cytochrome bc$_1$-COX complexes. A, mitochondria (50 µg of protein) isolated from WT, Δcox4, Δcyt1, and Δimp1 strains were analyzed by SDS-PAGE and Western blotting, and the steady state levels of ATP synthase subunits, Su e, Su g, or Atp4 and COX subunit Cox2 were analyzed using subunit-specific antisera, as indicated. Tim23 served as a loading control. B, digitonin solubilization and CN-PAGE analysis of the F$_{0}$/F$_{1}$-ATP synthase complexes was performed as described in Fig. 5A.

reported decrease in mitochondrial COX enzyme activity that is measured in the absence of the nonessential subunit of the ATP synthase, subunit g (Su g) (42). The results presented here confirmed this and also demonstrated a similar decrease in COX activity in mitochondria isolated from ΔSu e cells. Furthermore, we demonstrate here that the decrease in COX activity is not because of the indirect effect of instability of mtDNA maintenance and hence loss of coding capacity for essential COX subunits. In agreement, the analysis of the steady state levels of individual subunits of the COX complex showed that they are not significantly affected in the ΔSu g and ΔSu e mitochondria. Consistently, previous spectral analysis of the isolated ΔSu g mitochondria determined that there was no apparent decrease in the cytochrome content despite the measured reduction in COX enzyme activity (42). Thus, together we conclude that the observed reduction in the levels of COX enzyme activity is not because of an overall decrease in the COX protein levels in the absence of Su g (or Su e), but rather it reflects a change in an enzymatic property of the COX complex.

We report here that the higher ordered organizational state of the cytochrome bc$_1$-COX supercomplex is affected by the absence of Su e and Su g proteins. The perturbation in the assembly of the cytochrome bc$_1$-COX supercomplex was observed on two levels. First, a significant fraction of free cytochrome bc$_1$ complexes was detected in the ΔSu e and ΔSu g mitochondria, which, from their size and absence of co-migrating COX subunits, most likely correspond to dimeric and monomeric forms of the cytochrome bc$_1$ complex. Second, the remaining cytochrome bc$_1$ complexes present in the absence of Su e and/or Su g were in a supercomplex form with the COX complex (as indicated by co-migration with Cox2), which from its electrophoretic behavior appeared to be smaller than the cytochrome bc$_1$-COX supercomplex present in wild type mitochondria. Thus we conclude that the stoichiometry of the cytochrome bc$_1$-COX supercomplex is altered in the absence of the Su e and Su g proteins.

We observed that the Cox4 subunit displayed an altered association with the COX complex in the absence of Su g. Yeast Cox4 (equivalent to mammalian CoxVb) is an enzymatically essential subunit of the COX complex. Analysis of the crystal structure of the bovine COX complex indicates that Cox4/CoxVb is a peripherally associated subunit located on the matrix side of the COX complex (52). The altered assembly environment of Cox4, and also the observed stoichiometry changes in the cytochrome bc$_1$-COX supercomplex, may contribute toward the compromised COX activity observed in the ΔSu e and ΔSu g mitochondria.

The altered assembly status and stoichiometry of the cytochrome bc$_1$-COX supercomplex is unlikely to be simply because of a detergent artifact, i.e. increase instability of the complex during digitonin lysis. The solubilization and electrophoresis system used here is mild and retains the cytochrome bc$_1$-COX complex from the cardiolipin synthase mutant (Δcrd1) in an intact form, although it is known to exhibit a pronounced instability toward detergent (9). Furthermore, the measured decrease in COX enzyme activities in the intact ΔSu g mitochondria would also argue that the effect in the cytochrome bc$_1$-COX supercomplex in the absence of Su g (or Su e) is not because of an in vitro detergent artifact. Finally, Western blotting analysis of ΔSu g and ΔSu e mitochondria has indicated that the steady state levels of cytochrome c are markedly increased in the absence of Su g or Su e. An increased expression level of cytochrome c has been observed previously in other COX-mutant yeast strains (53, 54), further supporting the conclusion here that the COX activity is compromised in the absence of subunits e and g of the F$_{0}$/F$_{1}$-ATP synthase.

We report here that the oxidative phosphorylation state of the mitochondria did not have a direct effect on the assembly of the cytochrome bc$_1$-COX supercomplex. So although the ΔSu e and ΔSu g mitochondria have been reported to have a compromised membrane potential (24), this alone cannot account for the perturbation in the cytochrome bc$_1$-COX supercomplex assembly reported here. We should note here, however, that our independent membrane potential measurements performed with the ΔSu e and ΔSu g mitochondria used in this study (i.e. isolated from cells grown in the nonfermentable carbon source, glycerol) did not display a compromised membrane potential. The level of membrane potential measured in the ΔSu e and ΔSu g mitochondria was 84 and 94% of the wild type control, analyzed in parallel (results not shown).

The cytochrome bc$_1$-COX supercomplex assembled in the absence of Su g (and Su e, not shown) still retains the capacity to interact with a subpopulation of the TIM23 machinery, despite its apparent altered stoichiometry. In fact, we report here that increased levels of TIM23 complexes associated with the cytochrome bc$_1$-COX supercomplex was observed in detergent extracts from the ΔSu g and ΔSu e mitochondria. The association of the TIM23 complex with the cytochrome bc$_1$-COX supercomplex has been described to involve Tim21, a nonessential component of the TIM23 complex (46–49). This altered behavior of the TIM23 machinery in the absence of Su g and Su e cannot be simply attributed to altered Tim21 levels, as...
these are not elevated in the Δsu e and Δsu g mitochondria (results not shown). The reported lack of cristae membrane differentiation in the Δsu e and Δsu g null mitochondria may favor the association of TIM23 with the OXPHOS complexes, as the latter are normally enriched in the cristae membranes in wild type mitochondria (25–27).

How does the presence of ATP synthase subunits Su e and Su g influence the assembly state of the cytochrome bc1-COX supercomplex? In the absence of Su e and/or Su g, it has been shown that the ATP synthase still exists as dimers in the mitochondrial membrane; however, these dimers are not stable to detergent extraction. Thus the presence of Su e and/or Su g appears to be required for the stabilization, rather than the formation, of the ATP synthase dimers (6, 23, 30–37). We therefore consider it likely that it is the presence of the Su e and Su g proteins, rather than the dimeric state of the ATP synthase per se, that supports the organizational state of the cytochrome bc1-COX supercomplex. It is possible that the Su e and Su g proteins physically associate also with the cytochrome bc1-COX supercomplex, in addition to the ATP synthase, and by doing so bridge a physical relation between these OXPHOS complexes and thereby support their correct assembly. Indeed, in support of this possibility, preliminary cross-linking analysis indicates that the molecular environment of Su e within the intact mitochondrial membranes is altered in COX-deficient mitochondria.3 On the other hand, it is also possible that the alteration in the organizational state of the cytochrome bc1-COX supercomplex in the absence of Su e and Su g may be due to an indirect effect, for example as a result of the dramatically altered cristae membrane morphology, which has been reported to occur in the Δsu e and Δsu g mitochondria. Loss of cristae membrane architecture may therefore drastically affect the arrangement of these complexes (or specific lipids) within the inner membrane, which in turn may affect their assembly state and association with the TIM23 translocase.

In summary, we report here that the dimeric F1F0-ATP synthase can directly influence the organizational state of another OXPHOS complex, the cytochrome bc1-COX complex and its association with the TIM23 machinery. Our findings demonstrate that subunits Su e and Su g, in addition to their role in the dimerization of the ATP synthase complex, play an intimate role in the assembly state and activity of the cytochrome bc1-COX supercomplex and therefore highlight a further level of complexity in the organization of the mitochondrial OXPHOS supercomplexes.

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