F$_1$F$_\text{c}$-ATP synthase is a key enzyme of oxidative phosphorylation that is localized in the inner membrane of mitochondria. It uses the energy stored in the proton gradient across the inner mitochondrial membrane to catalyze the synthesis of ATP from ADP and phosphate. Dimeric and higher oligomeric forms of ATP synthase have been observed in mitochondria from various organisms. Oligomerization of ATP synthase is critical for the morphology of the inner mitochondrial membrane because it supports the generation of tubular cristae membrane domains. Association of individual F$_1$F$_\text{c}$-ATP synthase complexes is mediated by the membrane-embedded F$_\text{c}$-part. Several subunits were mapped to monomer-interfaces of yeast ATP synthase complexes, but only Su e (Atp21) and Su g (Atp20) have so far been identified as crucial for the formation of stable dimers. We show that two other small F$_\text{c}$-components, Su k (Atp19) and Su i (Atp18) are involved in the stepwise assembly of F$_1$F$_\text{c}$-ATP synthase dimers and oligomers. We have identified an intermediate form of the ATP synthase dimer, which accumulates in the absence of Su i. Moreover, our data indicate that Su i facilitates the incorporation of newly synthesized subunits into ATP synthase complexes.

INTRODUCTION

Mitochondria are ubiquitous organelles of eukaryotic cells that have evolved from endosymbiotic α-proteobacteria (Dolezal et al., 2006). The majority of the ~1000 different mitochondrial proteins are synthesized on cytosolic ribosomes and imported via specialized protein translocation machineries in a posttranslational manner (Neupert and Herrmann, 2007; Chacinska et al., 2009). Mitochondria play a key role in cellular energy metabolism, because they contain the metabolite carriers and enzymes involved in oxidative phosphorylation (Saraste, 1999; Palmieri et al., 2002). In this process, the proton-pumping protein complexes of the respiratory chain located in the inner mitochondrial membrane generate a proton-motive force that fuels ATP production by F$_1$F$_\text{c}$-ATP synthase. Respiratory chain complexes, such as the yeast cytochrome bc$_1$ complex and cytochrome c oxidase, are generally associated into different types of supercomplexes, also referred to as respirasomes (Schägger and Pfeiffer, 2000; Schägger, 2001; Wittig et al., 2006; Boekema and Braun, 2007; Acín-Pérez et al., 2008; Stuart, 2008; Vonck and Schäfer, 2009; Wittig and Schägger, 2009). These large structural arrangements were suggested to optimize electron transfer efficiency during respiration (Schägger, 2001; Wittig et al., 2006; Acín-Pérez et al., 2008; Wittig and Schägger, 2009). ATP synthase complexes have been found in monomeric, dimeric, and higher oligomeric forms in the inner mitochondrial membrane (Arnold et al., 1998; Schägger and Pfeiffer, 2000; Paumard et al., 2002a; Dudkina et al., 2005, 2006; Minauro-Sanmiguel et al., 2005; Bornhövd et al., 2006; Strauss et al., 2008; Thomas et al., 2008; Wittig et al., 2008). Respiratory chain supercomplexes accumulate together with F$_1$F$_\text{c}$-ATP synthase in the tubular cristae membrane domains that extend from the inner boundary membrane into the lumen of mitochondria (Gilkerson et al., 2003; Vogel et al., 2006; Wurm and Jakobs, 2006; Rabl et al., 2009; Zick et al., 2009).

The oligomeric state of F$_1$F$_\text{c}$-ATP synthase is a major determinant for the characteristic morphology of cristae membranes (Giraud et al., 2002; Paumard et al., 2002a; Arselin et al., 2004; Bornhövd et al., 2006; Rabl et al., 2009). Large chain-like assemblies of ATP synthase dimers were observed in the inner mitochondrial membrane (Allen et al., 1989; Buzhynskyy et al., 2007; Strauss et al., 2008). Such dimer ribbons have been proposed to induce membrane bending and eventually lead to the formation of cristae. Colocalization of membrane-bound respirasomes and F$_1$F$_\text{c}$-ATP synthase together with the tube-like shape of the cristae with sharp membrane bends at the apices is believed to generate a specific microenvironment that is beneficial for energy transduction reactions (Allen, 1995; Bornhövd et al., 2006; Strauss et al., 2008; Zick et al., 2009).

Mitochondrial F$_1$F$_\text{c}$-ATP synthase is composed of the rotor-domain, the catalytic centers in the F$_1$ head-domain and the peripheral stalk stator (Stock et al., 2000; Capaldi and Aeggeler, 2002; Fillingame et al., 2003; Weber and Senior, 2003; von Ballmoos et al., 2008). Proton flux through the interface of the membrane-embedded F$_\text{c}$-subunits Su 6 and Su 9 drives rotation of the rotor-domain that consists of a...
rieger et al. (1997; Dian et al., 2008). The peripheral stator stalk prevents rotation of $F_\alpha$ and $F_\beta$ together with the rotor domain. It is composed of the subunits b (Su b), d (Su d), and h (Su h), and the oligomycin sensitivity-conferring protein (Walker and Dickson, 2006).

The membrane-embedded $F_\gamma$-part of the yeast enzyme contains several small subunits, most of which are not essential for catalysis (Arnold et al., 1998; Velours et al., 2000; Stuart, 2008; Wittig and Schägger, 2008b). Two of these subunits, Su e (Atp21/Tim11) and Su g (Atp20), were shown to be critical for the stability of $F_1$-$F_0$-ATP synthase dimers (Arnold et al., 1998; Paumard et al., 2002a; Bornhövd et al., 2006; Wittig et al., 2008). Su e and Su g assemble with monomeric ATP synthase in a sequential manner to form a dimerization-competent primed monomer (Wagner et al., 2009). Deletion of the nuclear genes encoding Su e and Su g strongly inhibits dimer formation and leads to an aberrant, onion-like cristae morphology (Giraud et al., 2002; Paumard et al., 2002a; Arselin et al., 2004; Rabl et al., 2009). The small subunit Su k (Atp19) has been found associated only with dimeric ATP synthase (Arnold et al., 1998; Wittig and Schägger, 2008b). Su k was suggested to be peripherally attached to the $F_\gamma$-part on the intermembrane space side of the inner membrane via interactions with Su e and Su g (Arnold et al., 1998). The catalytically essential $F_\gamma$-subunits Su b (Atp4) and Su d (Atp18) as well as Su e (Atp18) and Su i (Atp14) were also localized to the interface between two ATP synthase monomers (Spannagel et al., 1998; Vaillier et al., 1999; Velours et al., 2000; Giraud et al., 2002; Paumard et al., 2002b; Soubannier et al., 2002; Fronzes et al., 2003, 2006; Gavin et al., 2005; Weimann et al., 2008; Wittig and Schägger, 2008b; Wittig et al., 2008). Depletion of Su h impairs the assembly of mitochondrially encoded Su 6 into ATP synthase and thus leads to a loss of function of the enzyme (Goyon et al., 2008). The molecular functions of Su k and Su i have remained largely unknown.

In this study, we have used a native gel assay to analyze the roles of Su k and Su i in the biogenesis of monomeric and dimeric $F_1$-$F_0$-ATP synthase. We find that Su k and Su i play an important role in the assembly and stabilization of mature ATP synthase dimers. Su i additionally promotes the incorporation of newlyimported subunits into monomeric ATP synthase complexes.

**In Vitro Import of Radiolabeled Precursor Proteins**

Polymerase chain reactions were carried out to amplify genes of interest fused to a SP6 promoter (Stojanovski et al., 2007). The mMESSAGE mMACHINE kit (Ambion, Austin, TX) and a rabbit reticulocyte lysate (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) were used for transcription and translation in the presence of [35S]methionine. Protein import reactions radiolabeled precursors were incubated with isolated mitochon-

**Blue Native Electrophoresis**

Mitochondria were solubilized by resuspending in digitonin buffer (0.35 or 1% [vol/vol] digitonin, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol, and 1 mM PMSF) and incubated for 30 min at 4°C. On centrifugation at 16,000 × g for 10 min at 4°C (clarifying spin) supernatants were mixed with 10% blue native sample buffer (100 mM Bis-Tris, pH 7.0, 5% [vol/vol] Coomassie Brilliant Blue G-250, and 500 mM N-epsilon-aminocapric acid). Depending on the required separation range, 3–13 or 4–8% (high-resolution) polyacrylamide blue native gradient gels were used. Western blot analysis was performed by transferring proteins on a polyvinylidene fluoride membrane. Antisera against Su g, Su e, Su k, Su i, Su h, Su b, Su g, and $F_\gamma$ were raised in rabbits using specific peptides derived from the amino acid sequence of the respective proteins. For immunodecoration these antibodies were used in combination with a horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO). Visualization of protein bands was achieved with the enhanced chemiluminescence detection system (GE Healthcare).

**Carbonate Extraction**

Carbonate extractions were performed to separate membrane integral from soluble proteins. Mitochondria were resuspended in 0.1 M sodium carbonate buffer, pH 10.8 (90% [vol/vol] 0.1 M Na2CO3, and 10% [vol/vol] 0.1 M NaHCO3). Samples were incubated on ice for 30 min and subsequently centrifuged for 60 min at 125,000 × g. Pellet (integral membrane proteins) and supernatant (soluble proteins) fractions were separated. As control total samples were treated equally but not subjected to ultracentrifugation. Total and supernatant samples were precipitated with trichloroacetic acid. All samples were subjected to SDS-PAGE and analyzed by immunoblotting.

**In Gel ATPase Activity Staining**

Visualization of $F_1$-$F_0$-ATP synthase complexes by in gel ATPase activity staining was performed according to Bornhövd et al. (2006), with slight modifications. After blue native electrophoresis gels were washed in H2O for 20 min. Gels were stained with 50 mM antimycin A, 8 mM oligomycin (AVO-mix) were added to the import reactions. After completion of import reactions mitochondria were treated with 50 μg/ml proteinase K for 15 min on ice to remove not imported precursors. Inhibition of proteinase K was achieved by the addition of 2 mM phenylmethylsulfonyl fluoride (PMSF). After washing in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS-KOH, pH 7.2), samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) or blue native (BN)-PAGE and analyzed by digital autoradiography.

**RESULTS**

**Two Dimeric Forms of $F_1$-$F_0$-ATP Synthase Differentially Associate with Su k**

Blue native and clear native PAGE have been widely used to study the oligomeric state of $F_1$-$F_0$-ATP synthase (Wittig and Schägger, 2008a). Depending on the type and concentration of the detergent used to solubilize mitochondrial membranes monomeric, dimeric and higher oligomeric forms of ATP synthase have been separated with these methods (Arnold et al., 1998; Schägger and Pfeiffer, 2000; Paumard et al., 2002a; Eubel et al., 2003; Gavin et al., 2005; Bornhövd et al., 2006; Dudkina et al., 2006; Wittig et al., 2008). To get more insight into the oligomeric organization of yeast $F_1$-$F_0$-ATP synthase, we have established conditions for blue native electrophoresis that allow the optimal separation of protein complexes in the high-molecular-weight range (high-resolution BN-PAGE; Wagner et al., 2009). We isolated mitochon-
dria from a wild-type *S. cerevisiae* strain, solubilized them under mild conditions in the presence of 1% digitonin, and subjected the protein extracts to high-resolution BN-PAGE. With antisera against the peripheral stalk subunits Su b and Su h and the small F0-subunit Su i, we detected two monomeric forms of F1F0-ATP synthase, M and M’ (Figure 1A, lanes 1–3). We have shown previously that the primed monomer M’ also contains Su e and Su g (Wagner et al., 2009). Interestingly, we also observed two different forms of the ATP synthase dimer with slightly different migration behavior, which we termed D and D’ (Figure 1A). Su b, Su h, and Su i (Figure 1A) as well as several other subunits of ATP synthase (data not shown; Wagner et al., 2009) were present in both dimeric forms. Only the small F0-subunit Su k was almost exclusively found in the faster migrating dimer D (Figure 1A, lane 4). In agreement with Arnold et al. (1998), Su k could not be detected in monomeric ATP synthase by immunoblotting in wild-type mitochondria.

To get a more dynamic picture of Su k assembly with F1F0-ATP synthase, we synthesized the precursor of Su k in an in vitro translation system in the presence of [35S]methionine. We incubated the radiolabeled precursor protein with isolated mitochondria and analyzed the incorporation of Su k into ATP synthase complexes by high-resolution BN-PAGE. Imported Su k assembled mainly into the faster migrating dimer D, but small amounts were also found in D’ and the monomeric ATP synthase forms M and M’ (Figure 1B, lanes 1 and 2). In mitochondria lacking the small F0-subunits Su e or Su g, very little or no dimeric F1F0-ATP synthase is found, whereas the monomeric form M accumulates (Arnold et al., 1998; Paumard et al., 2002a; Bornhövd et al., 2006; Wittig et al., 2008, Wagner et al., 2009). We isolated mitochondria from *atp20Δ* and *atp21Δ* yeast strains that are devoid of Su g or Su e, respectively, and imported radiolabeled Su k. As expected no assembly of Su k into dimeric ATP synthase was observed (Figure 1B, lanes 4–9). Instead, the amounts of Su k found in the monomer M were increased in *su gΔ* and *su eΔ* mutant mitochondria. Western blot analysis of digitonin-solubilized *su gΔ* and *su eΔ* mitochondria with antibodies against Su k confirmed that Su k accumulates in the monomer M in the absence of Su g and Su e (Figure 1C). These data indicate that newly synthesized Su k preferentially assembles into dimeric F1F0-ATP synthase but also associates with the monomeric form of the enzyme. It has been shown that Su g is not stably expressed in the absence of Su e meaning that *su eΔ* mitochondria are devoid of both, Su e and Su g (Arnold et al., 1998; Wagner et al., 2009). Thus, binding of Su k to monomeric ATP synthase does not depend on Su e and Su g.

We observed that assembly of Su k with F1F0-ATP synthase is strictly dependent on the electrical potential across the inner mitochondrial membrane (Δψ). When Δψ was inhibited by the addition of antimycin A, valinomycin, and oligomycin before the import reaction, no incorporation of Su k into ATP synthase complexes was observed (Figure 1B, lanes 3, 6, and 9). Analysis of Su k import reactions by SDS-PAGE revealed that not only assembly into native complexes but also import of Su k to a protease-protected location within mitochondria was blocked when Δψ was inhibited (Figure 1D). This was a surprising finding because Su k was shown to be a peripheral membrane protein attached to the intermembrane space side of ATP synthase. Different pathways for protein import into the intermembrane space have been described, and only one of them requires the Δψ across the inner membrane (Herrmann and Hell, 2005). In this pathway, proteins are initially inserted into the inner membrane via the TIM23 machinery by a stop-transfer mechanism and the mature protein is subsequently released into the intermembrane space by proteolytic cleavage (Glick

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**Figure 1.** Su k differentially associates with different forms of F1F0-ATP synthase. (A) Mitochondria isolated from a wild-type (WT) *S. cerevisiae* strain were solubilized in 1% digitonin, and protein complexes were separated on a 4–8% polyacrylamide blue native gel. Membrane-integral and soluble proteins were separated by ultracentrifugation (125,000 × g). Total (T), pellet (P), and supernatant (S) fractions were analyzed by SDS-PAGE and Western blotting with the indicated antibodies. (B) WT, *su gΔ*, and *su eΔ* mutant mitochondria were analyzed by BN-PAGE and digital autoradiography. (C) WT, *su eΔ*, and *su gΔ* mutant mitochondria were solubilized in digitonin-containing buffer and analyzed by BN-PAGE and digital autoradiography. (D) Mitochondria were incubated with radiolabeled Su k in the presence or absence of Δψ. On treatment with proteinase K mitochondria were solubilized in digitonin-containing buffer and analyzed by BN-PAGE and digital autoradiography.
Su k is required for the formation of stable ATP synthase dimers. (A) Analysis of steady-state protein levels of wild-type (WT) and su kΔ mitochondria. Ten, 20, and 30 μg of mitochondrial proteins were analyzed by SDS-PAGE and Western blotting. (B) Isolated wild-type (WT) and su kΔ mitochondria were solubilized in buffer containing 1% digitonin and analyzed by BN-PAGE and immunoblotting with the indicated antisera. M, monomeric F1Fo-ATP synthase; M′, primed monomer; D′ and D, dimeric forms of F1Fo-ATP synthase.

Su k Stabilizes Dimeric Forms of ATP Synthase
We then asked whether Su k is involved in the dimerization of F1Fo-ATP synthase. We constructed a yeast strain that lacks the nuclear gene encoding Su k (ATP19). Deletion of ATP19 had no significant effect on the growth of yeast cells on nonfermentable medium (YPG) (Supplemental Figure 1A). We isolated mitochondria from Su k-deficient and corresponding wild-type cells and compared the steady-state levels of different mitochondrial proteins. All proteins tested including the catalytically essential ATP synthase subunits F1β, F1γ, and Su b; the small F0-subunits Su e, Su g, and Su i; and the control proteins cytochrome c1 (Cyt1), Cor1, and Tom40 were present in equal amounts in wild-type and su kΔ mitochondria (Figure 2A). ATP-dependent import of the radiolabeled F1β precursor protein into su kΔ mitochondria was comparable to wild-type (Supplemental Figure 1B, lanes 1–5 and 11–15). On solubilization in digitonin buffer, we analyzed the oligomeric state of ATP synthase in wild-type and su kΔ mitochondria by high-resolution BN-PAGE and immunoblotting. Using antibodies against the peripheral stalk subunit Su h and Su b, the F1-component F1β and the small F0-subunits Su g, Su e, and Su i, we detected strongly reduced amounts of ATP synthase dimers in su kΔ mitochondria, whereas the amounts of the monomeric forms were increased (Figure 2B). These data indicate that Su k is required for the formation of stable F1Fo-ATP synthase dimers. To directly monitor the assembly of newly synthesized subunits into monomeric and dimeric ATP synthase complexes, we imported different radiolabeled precursors into wild-type and su kΔ mitochondria and analyzed the assembly of these proteins with F1Fo-ATP synthase. Incorporation of the central stalk subunit F1γ (Figure 3A) and the peripheral stalk component Su h (Figure 3B) into the monomeric forms M and M′ was independent of Su k. In contrast, very little assembly of F1γ and Su h with dimeric ATP synthase was observed in su kΔ mitochondria, reflecting the reduced steady-state levels of dimers (compare Figures 2B and 3, A and B). On import into su kΔ mitochondria, radiolabeled Su e and Su g accumulated in the primed monomer M′, but virtually no ATP synthase dimers were detected (Figure 3, C and D). Thus, the primed monomer M′ is still formed in su kΔ mitochondria, because the assembly of Su e and Su g with monomeric ATP synthase is independent of Su k. One or more subsequent steps in the formation of stable F1Fo-ATP synthase dimers are impaired in the absence of Su k.

When low concentrations of digitonin are used for extraction of mitochondrial membrane protein complexes, higher oligomeric forms of F1Fo-ATP synthase remain intact. Such larger oligomers have been visualized in native gels by means of ATPase activity staining (Paumard et al., 2002a; Arselin et al., 2004; Bornhövd et al., 2006; Strauss et al., 2008; Weimann et al., 2008; Wittig et al., 2008). We solubilized mitochondria in buffer containing 0.35% digitonin and examined the protein extracts on 3–13% blue native gels. Using an activity staining based on calcium phosphate precipitation upon ATP hydrolysis, we detected not only monomers and dimers but also a higher oligomeric form of ATP synthase (Figure 4A, lane 1). We used these particularly mild solubilization conditions to investigate import and assembly of radiolabeled Su h in wild-type and su kΔ mitochondria. In wild-type mitochondria, Su h was incorporated into all detectable forms of ATP synthase in a membrane potential-dependent manner, indicating that assembly of newly imported subunits is also possible in the higher oligomeric states (Figure 4A, lanes 2–4). In su kΔ mitochondria assembly of Su h into dimers and oligomers of F1Fo-ATP synthase...
was clearly reduced, whereas an increased amount of imported Su h was found in the monomers (Figure 4A, lane 5–7). This altered ratio reflects the reduced stability of dimers and oligomers in the absence of Su k. An additional indication for the instability of ATP synthase oligomers in su k/H9004 mitochondria is the accumulation of an intermediate-size complex form running between oligomers and dimers on the native gel (Figure 4A, lane 6, marked with an arrowhead), which probably results from a partial fragmentation of oligomers. For comparison, we analyzed import of Su h into mitochondria from strains lacking the small subunits Su g and Su e under the same conditions. In both su g/H9004 and su e/H9004 mitochondria, virtually no assembly of Su h into ATP synthase dimers and oligomers was observed, whereas incorporation into the monomer was more pronounced (Figure 4B). Thus, lack of Su g or Su e, which are known to be central to the formation of stable dimers, has a more severe impact on the oligomeric state of F1Fo-ATP synthase compared with lack of Su k. We conclude that Su k is not essential for the formation but is important for the stability of F1Fo-ATP synthase dimers and oligomers.

Formation of One Dimeric Form of ATP Synthase Is Inhibited in the Absence of Su i

Given the results obtained for Su k, we asked whether Su i, another small peripheral subunit of F1Fo-ATP synthase that has been mapped to the monomer–monomer interface, is also required for the formation of stable dimers. We constructed a yeast strain, in which the nuclear gene encoding Su i (ATP18) was replaced by a selectable marker. Growth of the yeast strain lacking Su i (atp18/H9004) on nonfermentative glycerol medium (YPG) was similar to wild-type at 30°C but was significantly impaired at elevated temperatures (Supplemental Figure 1A). To minimize secondary effects, we therefore isolated mitochondria from wild-type and Su i-deficient cells grown at 30°C and compared the steady-state protein levels by SDS-PAGE and Western blotting. Subunits of the F1-part of ATP synthase (F1β and F1γ), components of the F1-sector, such as the catalytic subunit Su 6; the small subunit of the F1-sector Su 7; and the small subunit of the Fo-sector Su 6, were detected by Western blotting.
subunits \( \text{Su}_g \), \( \text{Su}_e \), and \( \text{Su}_k \); and control proteins, such as the cytochrome \( \text{bc}_1 \) complex subunit Cor1 and the outer membrane protein Tom40, were present in \( \text{su}^{-} \) mitochondria in equal amounts compared with wild-type mitochondria (Figure 5A). Both the ATP-dependent import of the precursor of \( \text{F}_1 \) (Supplemental Figure 1B, lanes 1–10) and the membrane potential across the inner mitochondrial membrane (Supplemental Figure 1C) were similar in wild-type and \( \text{su}^{-} \) mitochondria, indicating that the overall energy state was comparable. We solubilized wild-type and \( \text{su}^{-} \) mitochondria in buffer containing 1% digitonin and analyzed protein complexes by high-resolution BN-PAGE. Lack of \( \text{Su}_i \) had profound effects on the different \( \text{F}_1\text{F}_0\text{-ATP synthase} \) complex forms. Using antibodies against \( \text{F}_1 \), the central stalk component \( \text{F}_1 \), the peripheral stalk subunits \( \text{Su}_b \) and \( \text{Su}_h \) or the small peripheral \( \text{F}_0 \)-subunits \( \text{Su}_g \) and \( \text{Su}_e \), we observed much less dimeric \( \text{ATP synthase} \) in \( \text{su}^{-} \) mitochondria, whereas the amount of monomers was increased (Figure 5B, lanes 1–12). Strikingly, the effects on the two distinct dimeric forms (Figure 1A) were very different. Whereas the amounts of the faster migrating form \( D \) were strongly reduced, the slower migrating dimer \( D' \) was observed at normal levels or appeared even more abundant in \( \text{su}^{-} \) mitochondria compared with the wild-type situation (Figure 5B, lanes 1–12). Thus, in the absence of \( \text{Su}_i \) the dimer \( D' \) is still formed, whereas generation of \( D \) is inhibited. As shown in Figure 1A, \( \text{Su}_k \) was almost exclusively detected in the faster migrating dimer \( D \) by Western blotting in wild-type mitochondria. In agreement with this finding, the amount of \( \text{Su}_k \) bound to \( \text{F}_1\text{F}_0\text{-ATP synthase} \) was strongly

Figure 5. \( \text{Su}_i \) is required for the formation of a distinct \( \text{F}_1\text{F}_0\text{-ATP synthase} \) dimer form. (A) Analysis of steady-state protein levels in wild-type (WT) and \( \text{su}^{-} \) mitochondria. Ten, 20, and 30 \( \mu \)g of mitochondrial proteins were analyzed by SDS-PAGE and immunoblotting. (B) Digitonin-solubilized wild-type (WT) and \( \text{su}^{-} \) mitochondria were subjected to high-resolution BN-PAGE and analyzed by Western blotting with the indicated antisera. (C) Isolated WT, \( \text{Su}_g \) S62E, and \( \text{Su}_g \) S62A mitochondria were solubilized in buffer containing 1% digitonin. Protein complexes were separated on 4–8% polyacrylamide blue native gels and analyzed by immunoblotting with antisera against \( \text{F}_1\beta \), \( \text{Su}_9 \) and \( \text{Su}_k \). M, monomeric \( \text{ATP synthase} \); \( M' \), primed monomer; \( D' \) and \( D \), dimeric forms of \( \text{F}_1\text{F}_0\text{-ATP synthase} \); \( F_1 \), detached \( \text{F}_1\)-subcomplex.

Figure 6. Independent assembly of \( \text{Su}_i \) and \( \text{Su}_e/g \) with monomeric \( \text{ATP synthase} \). (A) Wild-type (WT), \( \text{su}^{g} \), and \( \text{su}^{e} \) mitochondria were incubated with \( ^{35}\text{S}\)-labeled \( \text{Su}_i \) in the presence or absence of a \( \Delta \mu \). Subsequent to proteinase K treatment, mitochondria were solubilized in digitonin-containing buffer. Samples were applied to a 4–8% blue native gel and protein complexes were visualized by digital autoradiography. (B and C) Radiolabeled \( \text{Su}_e \) and \( \text{Su}_g \) were imported into WT and \( \text{su}^{-} \) mitochondria. Samples were analyzed as described in A. M, monomeric \( \text{ATP synthase} \); \( M' \), primed monomer; \( D' \) and \( D \), dimeric forms of \( \text{F}_1\text{F}_0\text{-ATP synthase} \).
reduced in su iΔ mitochondria, where the D-form was impaired (Figure 5B, lanes 13 and 14). Moreover, we observed an increased amount of an ~450-kDa complex in su iΔ mitochondria that was observed with antibodies against F1β and F1γ (Figure 5B, lanes 1–4) but not with antibodies against the F1α-subunits Su b and Su h (Figure 5B, lanes 5–8). Therefore, this complex species probably represents a detached F1-subcomplex indicating that the interaction between F1- and Fo-portions of ATP synthase is slightly weakened in the absence of Su i (Paumard et al., 2002a).

In a previous study, we have shown that phosphorylation of Su g at serine 62 influences the monomer–dimer equilibrium of F1Fo-ATP synthase (Reinders et al., 2007). When serine 62 is replaced by glutamate, mimicking a constitutive phosphorylation of this residue, a reduced amount of dimeric ATP synthase was observed, whereas the relative abundance of the monomer was increased. Replacement of serine 62 by alanine, which cannot be phosphorylated, led to a slight increase in the relative amount of dimers. Our newly established high-resolution blue native electrophoresis conditions allow us to separate two different forms of dimeric ATP synthase (Figure 1A). In light of the specific role of Su i for the formation of one distinct dimer, we analyzed digitonin-solubilized wild type, Su g S62E, and Su g S62A mitochondria by high-resolution BN-PAGE by using antibodies against the F1α-protein F1β and the F1α-subunit Su 9 (Figure 5C, lanes 1–6). Remarkably, the phosphomimicking mutation Su g S62E induced a strong reduction of the amounts of the faster migrating dimer D, whereas the slower migrating dimer D' was present and even more abundant compared with the wild-type situation (Figure 5C, lanes 1, 2, 4, and 5). (It should be noted that the low amounts of D' in wild-type mitochondria are due to the growth of cells in selective minimal medium for this particular experiment (see Materials and Methods). Under these growth conditions, the levels of D' are close to the detection limit in wild-type mitochondria). As also seen in su iΔ mitochondria, association of Su k with ATP synthase complexes was impaired in Su g S62E mitochondria (Figure 5C, lanes 7 and 8). We conclude that lack of Su i and phosphorylation of Su g have very similar effects on ATP synthase dimer formation.

To examine the functional link between Su i and Su g in more detail, we analyzed import and assembly of the radiolabeled precursor of Su i by high-resolution BN-PAGE. In wild-type mitochondria, Su i was incorporated into the monomer M and the primed monomer M' as well as into the dimeric ATP synthase forms (Figure 6A, lanes 1–3). In both su gΔ and su eΔ mitochondria, where the monomer M accumulates (Wagner et al., 2009), Su i was efficiently incorporated into the M-form (Figure 6A, lanes 4–9), indicating that assembly of Su i with monomeric ATP synthase is independent of Su g and Su e. We then imported the radiolabeled precursors of Su e and Su g into wild-type and su iΔ mutant mitochondria. Su e and Su g assembled with monomeric F1Fo-ATP synthase to form the primed monomer M', which triggers the formation of dimeric complex forms in wild-type mitochondria (Figure 6, B and C, lanes 1–3; Wagner et al., 2009). In su iΔ mitochondria, where dimer formation is impaired, Su e and Su g were still able to assemble efficiently with monomeric ATP synthase to generate M’ (Figure 6, B and C, lanes 4–6). This shows that the association of Su e and Su g with ATP synthase does not require Su i. In conclusion, incorporation of Su i and the sequential assem-

Figure 7. Su i facilitates the incorporation of imported subunits into central and peripheral stalk regions. 35S-Labeled F1γ (A), F1δ (B), and Su h (C) were imported into wild-type (WT) and su iΔ mitochondria in the presence or absence of a Δψ. On treatment with proteinase K and solubilization of mitochondria in digitonin buffer, samples were subjected to BN-PAGE (A–C, top) or SDS-PAGE (A–C, bottom). Visualization of imported proteins was done by digital autoradiography. M, monomeric ATP synthase; M’, primed monomer; D’ and D, dimeric forms of F1Fo-ATP synthase; F1−, F1−subcomplex.
bly of Su e and Su g with F1F0-ATP synthase are independent events.

Su i Facilitates the Assembly of Imported Subunits into Monomeric F1F0-ATP Synthase

We then asked how the absence of Su i affects the assembly of other newly imported subunits into the different F1F0-ATP synthase forms. We synthesized the radiolabeled precursors of several subunits of F1F0-ATP synthase in vitro and incubated them with wild-type and su i Δ mutant mitochondria (Figure 7). On analysis by high-resolution BN-PAGE, we observed only very little incorporation of the central stalk components F1γ (Figure 7A, lanes 4–6) and F1δ (Figure 7B, lanes 4–6) as well as the peripheral stalk protein Su h (Figure 7C, lanes 4–6) into ATP synthase dimers, when Su i was missing. This can be explained by the strongly reduced levels in su i Δ mutant mitochondria of the dimeric form D, which is very abundant in wild-type mitochondria. Surprisingly, however, efficient assembly of individual subunits into ATP synthase monomers also was impaired in the absence of Su i. The degree of inhibition varied with the type of precursor analyzed. For example, F1γ showed only slightly reduced assembly with monomeric ATP synthase in su i Δ compared with wild-type mitochondria (Figure 7A, lanes 1–6), whereas the effects on the incorporation of F1δ (Figure 7B, lanes 1–6) and Su h (Figure 7C, lanes 1–6) were clearly more pronounced. Control experiments, in which the import reactions were analyzed by SDS-PAGE, revealed that the efficiency of import to a protease-protected location was comparable in wild-type and su i Δ mitochondria for all precursors tested (Figure 7, A–C, lanes 7–16). This means that mitochondria lacking Su i are specifically impaired in the assembly of several newly synthesized precursor proteins with F1F0-ATP synthase.

Assembly of in vitro-synthesized, radiolabeled Su k into dimeric as well as monomeric ATP synthase complexes was almost completely blocked in su i Δ mitochondria (Figure 8A, lanes 1–6), although import of Su k to a protease-protected location was comparable to wild-type mitochondria (Figure 8A, lanes 7–14). Hence, accumulation of Su k in the monomeric form, as observed in mitochondria that do not form stable ATP synthase dimers due to lack of Su g or Su e (Figure 1B), does not occur when dimerization is inhibited by the absence of Su i. The radiolabeled, imported precursor of the peripheral stalk subunit Su b exclusively assembled into the smaller monomeric F1F0-ATP synthase complex M in wild-type mitochondria (Figure 8B, lanes 1–3) that is not capable of dimerization (Wagner et al., 2009). Assembly is therefore not affected by the absence of Su g or Su e, which are only present in the dimerization-competent primed monomer M′ (Figure 8C). In su i Δ mitochondria assembly of newly synthesized Su b with ATP synthase was undetectable (Figure 8B, lanes 4–6), although import of the Su b precursor to a protease-protected location within mitochondria and processing of the N-terminal signal sequence occurred with similar efficiency as in wild-type mitochondria (Figure 8B, lanes 7–14). We conclude that Su k and Su b show the most pronounced dependence on Su i for incorporation into preexisting ATP synthase complexes.

DISCUSSION

Dimerization and subsequent oligomerization of F1F0-ATP synthase is an important driving force for the formation of the tubular cristae domains of the inner mitochondrial membrane. The characteristic structure of cristae membranes provides an optimal environment for the energy-transducing

Figure 8. Su i is required for the assembly of newly synthesized Su k and Su b with F1F0-ATP synthase. (A) Wild-type (WT) and su i Δ mutant mitochondria were incubated with the radiolabeled precursor of Su k in the presence or absence of a Δψ. After proteinase K-treatment, mitochondria were either solubilized in digitonin-containing buffer and analyzed by BN-PAGE (top) or denatured for analysis by SDS-PAGE (bottom). Digital autoradiography was used for the detection of imported proteins. (B) Import of 35S-labeled Su b was carried out and analyzed as described in A. (C) 35S-Labeled Su b was imported into WT, su gΔ, and su eΔ mitochondria and analyzed by BN-PAGE and digital autoradiography as described in A. M, monomeric F1F0-ATP synthase; M′, primed monomer; D′ and D, dimeric forms of ATP synthase.
reactions involved in oxidative phosphorylation (Allen, 1995; Gilkerson et al., 2003; Vogel et al., 2006; Stuart, 2008; Strauss et al., 2008; Zick et al., 2009). Current models for ATP synthase dimerization and oligomerization highlight the roles of the small peripheral F$_o$-subunits Su e and Su g in this process (Stuart, 2008; Wittig and Schägger, 2008b). Cross-linking studies and fluorescence energy transfer experiments have indicated that the monomer-monomer interfaces of F$_o$F$_{1}$-ATP synthase complexes involve several other proteins (Spannagel et al., 1998; Velours et al., 2000; Giraud et al., 2002; Paumard et al., 2002b; Gavin et al., 2005; Fronzes et al., 2006; Weimann et al., 2008). Our study sheds a new light on the intricate molecular mechanisms that control the oligomeric state of F$_o$F$_{1}$-ATP synthase. We show for the first time that two small F$_o$-subunits, Su k and Su i, contribute to the generation of dimeric and higher oligomeric forms of the enzyme. Whereas the association of Su g with ATP synthase monomers depends on Su e (Wagner et al., 2009), incorporation of Su k requires Su i. We demonstrate that Su e/g and Su i/k form distinct modules, which are involved in different steps of the formation of dimeric ATP synthase. Su e/g are needed for the generation of a primed monomer that is competent for dimerization. In the absence of Su i/k, the primed monomer is still formed, but dimer formation is inhibited indicating that Su i/k act mainly downstream of Su e/g. We conclude that at least four subunits of the membrane-embedded F$_o$-sector, which are dispensable for ATP synthesis per se, are required for the formation of stable F$_o$F$_{1}$-ATP synthase dimers: Su e, Su g, Su k, and Su i. Apart from these stoichiometric subunits of ATP synthase, other inner membrane components, such as the dynamin-like GTPase Mgm1 (Amutha et al., 2004) and the putative cristae junction protein Fcj1 (Rabl et al., 2009), also have been proposed to influence the oligomeric state of ATP synthase by yet unknown mechanisms. Hence, multiple factors control the oligomeric state of ATP synthase and influence the morphogenesis of cristae membranes.

We propose that Su i also has a role for monomeric ATP synthase. Our data indicate that Su i facilitates the incorporation of newly synthesized imported subunits into the central and peripheral stalk regions of preexisting ATP synthase complexes. The association of single imported components into large protein complexes probably occurs via subunit exchange (Daley, 2008). Such regulated turnover requires a substantial dynamic flexibility of protein complexes. The small ATP synthase subunit Su i seems to be important for achieving this conformational flexibility. This notion is exemplified by the strong Su i-dependence of Su b assembly with ATP synthase. Cross-linking studies and electron microscopy images indicate that Su b is sandwiched between the two monomers at the dimer interface (Spannagel et al., 1998; Dudkina et al., 2005; Minauro-Sanmiguel et al., 2005; Weimann et al., 2008; Wittig and Schägger, 2009). In wild-type mitochondria, Su b can therefore only be incorporated into the monomer M that is devoid of Su e and Su g and not competent for dimerization. In the absence of Su i, however, assembly of imported Su b with the monomer M could not be detected. In conclusion, Su i is not only required for the incorporation of new subunits into F$_o$F$_{1}$-ATP synthase already at the level of the monomeric form. Beige, inner mitochondrial membrane; blue, F$_{1}$-part of ATP synthase; light green, F$_{o}$-part of ATP synthase with peripheral stalk; dark green, Su i at the monomeric ATP synthase facilitating subunit exchange; red, Su e and Su g; yellow, Su i mediating the transition from D’ to D; and orange, Su k.

Figure 9. Model for stepwise assembly of dimeric F$_o$F$_{1}$-ATP synthase in mitochondria. Sequential association of Su e and Su g with monomeric ATP synthase (M) leads to the formation of a primed monomer (M’). An intermediate dimeric form (D’) assembles from these primed monomers. Su i drives the conversion of the intermediate dimer to the mature dimer (D), which stably associates with Su k. In addition to its role in dimer maturation, Su i facilitates the
formation of stable ATP synthase dimers but also supports the exchange of subunits in the monomeric state.

We have identified two distinct forms of F_{1}F_{0}-ATP synthase dimers, D and D'. Earlier studies have provided initial indications that more than one type of ATP synthase dimer may exist, but their molecular identities and functions have remained enigmatic (Paumard et al., 2002a; Arselin et al., 2004). By single particle electron microscopy different ATP synthase dimers with distinct geometrical shapes were observed. These distinct dimer shapes were suggested to reflect the association of monomers via different interaction surfaces (Dudkina et al., 2006; Strauss et al., 2008). It could be possible that the two dimeric forms we observe represent dimers with front-to-front and side-to-side association of monomers, respectively. This might then explain, why the lack of Su i impairs the formation of only one distinct dimeric form, the D-form. However, the results of our native assembly assay, which allows us to directly monitor the incorporation of imported subunits into ATP synthase, favor an intriguing alternative explanation. We show that in the absence of Su i, only the D'-form is efficiently generated. Moreover Su i is required for the stable incorporation of Su k into ATP synthase. Therefore, we conclude that the association of Su e and Su g with the monomer M is sufficient for the formation of D'. At steady state, Su k is firmly associated only with the D-form of F_{1}F_{0}-ATP synthase. Together, these data suggest that D' is an intermediate form of the dimeric F_{1}F_{0}-ATP synthase. Su i is required for the transition from D' to the mature dimer D. This transition then allows stable binding of Su k with ATP synthase. The reduced levels of both D' and D in su-Δk mitochondria suggest that the Su i-driven rearrangements in D' still take place, but the mature dimer D is not stable without Su k. Based on these findings, we propose the following model for the assembly of dimeric F_{1}F_{0}-ATP synthase (Figure 9). 1) Su e and Su g bind in a sequential manner to the monomer M generating the primed monomer M'. 2) Association of primed monomers leads to the formation of the intermediate dimer D'. 3) D' subsequently undergoes a conformational rearrangement resulting in the formation of the mature dimer D, which is stabilized by the addition of Su k. Two factors are critical for the transition from the intermediate dimer D' to the mature dimer D: the presence of Su i and the phosphorylation state of Su g. Phosphorylation of Su g and absence of Su i both inhibit the formation of D. We therefore suggest that reversible phosphorylation of Su g reduces the relative amount of F_{1}F_{0}-ATP synthase dimers by negatively regulating the dimer-promoting activity of Su i. The faster migration of the mature dimer D on high-resolution blue native gels may be caused by a different, more compact conformation or by alterations in the stoichiometry of the small F_{0}-subunits.

Together, our study indicates that the biogenesis of stable F_{1}F_{0}-ATP synthase dimers from monomeric complexes is a stepwise process that involves two intermediate stages, M' and D'. Whereas Su e and Su g are crucial for the formation of M', Su i is important for the generation of the mature dimer form D from D'. Su k tightly binds to the mature dimer and stabilizes this complex. Su i-dependent maturation of ATP synthase dimers and stabilization by Su k support the formation of larger oligomeric forms, which are important for the generation of tubular cristae membrane domains.

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