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Stepwise Assembly of Dimeric F_1F_o-ATP Synthase in Mitochondria Involves the Small F_o-Subunits k and i

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F_1F_o-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_1F_o-ATP synthase complexes is mediated by the membrane-embedded F_o-part. Several subunits were mapped to monomer-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_o-components, Su k (Atp19) and Su i (Atp18) are involved in the stepwise assembly of F_1F_o-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*., 2006). 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_1F_o-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_1F_o-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_1F_o-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_1F_o-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_1F_o-ATP synthase is composed of the rotor-domain, the catalytic centers in the F_1 head-domain and the peripheral stator stalk (Stock *et al*., 2000; Capaldi and Aggeler, 2002; Fillingame *et al*., 2003; Weber and Senior, 2003; von Ballmoos *et al*., 2008). Proton flux through the interface of the membrane-embedded F_o-subunits Su 6 and Su 9 drives rotation of the rotor-domain that consists of a

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Abbreviations used: $\Delta\psi$, membrane potential; $F_1\beta$, $F_1\gamma$, $F_1\delta$: β, γ, and δ subunits of F_1F_o-ATP synthase, respectively; Su i, subunit i (Atp18) of F_1F_o-ATP synthase, Su k, subunit k (Atp19) of F_1F_o-ATP synthase.

ring-shaped oligomer of Su 9 and the F_1-components $F_1\gamma$, $F_1\delta$, and $F_1\varepsilon$. $F_1\gamma$ connects the Su 9-ring with the catalytic subunits $F_1\alpha$ and $F_1\beta$ and converts rotary motion into conformational changes in the nucleotide-binding pockets (Noji *et al*., 1997; Dian *et al*., 2008). The peripheral stator stalk prevents rotation of $F_1\alpha$ and $F_1\beta$ 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_o-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_1F_o-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_o-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_o-subunits Su b (Atp4) and Su 6, as well as Su i (Atp18) and Su h (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_1F_o-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 newly imported subunits into monomeric ATP synthase complexes.

MATERIALS AND METHODS

Yeast Strains, Growth Conditions, and Isolation of Mitochondria

All yeast strains used in this study are derivatives of the *Saccharomyces cerevisiae* strain YPH499. Deletion of the *ATP18* and *ATP19* genes encoding Su i and Su k, respectively, was achieved by homologous recombination of a *His3MX6* cassette into the corresponding locus (Longtine *et al*., 1998). Strains *atp20*Δ (lacking Su g) and *atp21*Δ (lacking Su e) have been described previously (Wagner *et al*., 2009). For preparation of mitochondria, yeast deletion mutants and the corresponding wild-type cells were grown in YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, and 3% [vol/vol] glycerol) at 30°C. Yeast strains containing plasmids pRS413-Su g (wild-type), pRS413-Su g-S62E, or pRS413-Su g-S62A in a *atp20*Δ background (Reinders *et al*., 2007) were grown at 24°C on selective medium in the presence of 3% [vol/vol] glycerol and 0.3% [wt/vol] glucose. Mitochondria were isolated by differential centrifugation as described previously (Stojanovski *et al*., 2007). Membrane potential of isolated mitochondria was determined as published previously (Geissler *et al*., 2000).

In Vitro Import of Radiolabeled Precursor Proteins

Polymerase chain reactions were carried out to amplify genes of interest fused to a SP6 promotor (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 [^{35}S]methionine, respectively. For protein import reactions radiolabeled precursors were incubated with isolated mitochondria in import buffer (3% [wt/vol] bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM methionine, 10 mM MOPS-KOH, pH 7.2), in the presence of 2 mM ATP and 2 mM NADH. For dissipation of the membrane potential 1 μM valinomycin, 8 μM antimycin A, and 20 μM 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.

Blue Native Electrophoresis

Mitochondria were solubilized by resuspending in digitonin buffer (0.35 or 1% [wt/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 $\times$ *g* for 10 min at 4°C (clarifying spin) supernatants were mixed with 10$\times$ blue native sample buffer (100 mM Bis-Tris, pH 7.0, 5% [wt/vol] Coomassie Brilliant Blue G-250, and 500 mM ε-amino *n*-caproic 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 9, and $F_1\gamma$ 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 Na_2CO_3 and 10% [vol/vol] 0.1 M $NaHCO_3$). Samples were incubated on ice for 30 min and subsequently centrifuged for 60 min at 125,000 $\times$ *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_1F_o-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 H_2O for 20 min. Gels were incubated in an ATP-containing buffer (50 mM glycine, pH 8.4, 5 mM $MgCl_2$, and 20 mM ATP) for 20 min and subsequently transferred into a 10% $CaCl_2$ solution. Staining was stopped in H_2O after the appearance of calcium phosphate precipitates in the gel.

RESULTS

Two Dimeric Forms of F_1F_o-ATP Synthase Differentially Associate with Su k

Blue native and clear native PAGE have been widely used to study the oligomeric state of F_1F_o-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_1F_o-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 F_o-subunit Su i, we detected two monomeric forms of F_1F_o-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 F_o-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 F_1F_o-ATP synthase, we synthesized the precursor of Su k in an in vitro translation system in the presence of [^{35}S]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 F_o-subunits Su e or Su g, very little or no dimeric F_1F_o-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 *atp20*Δ and *atp21*Δ 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 g*Δ and *su e*Δ mutant mitochondria. Western blot analysis of digitonin-solubilized *su g*Δ and *su e*Δ 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 F_1F_o-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 e*Δ 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 F_1F_o-ATP synthase is strictly dependent on the electrical potential across the inner mitochondrial membrane ($\Delta\psi$). When $\Delta\psi$ 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 $\Delta\psi$ 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 $\Delta\psi$ 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

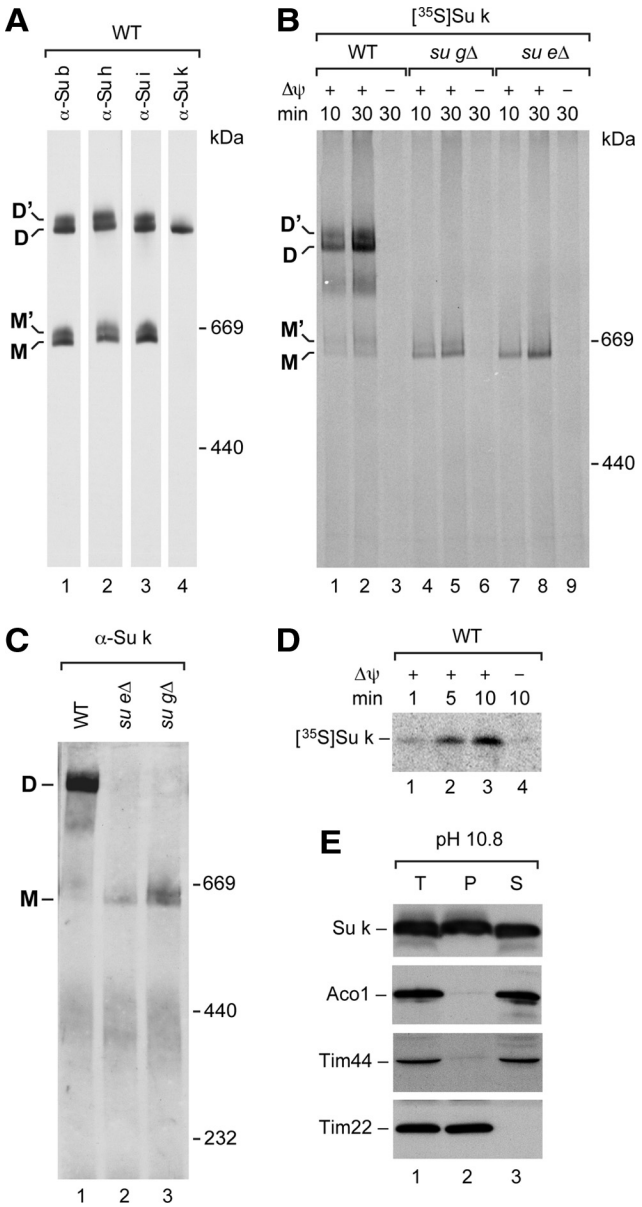

Figure 1. Su k differentially associates with different forms of F_1F_o-ATP synthase. (A) Mitochondria isolated from a wild-type (WT) *S. cerevisiae* strain were solubilized in 1% digitonin, protein complexes were separated on a 4–8% polyacrylamide blue native gel and analyzed by immunoblotting with the indicated antisera. (B) ^{35}S-Labeled Su k was imported into isolated wild-type (WT), *su g*Δ, and *su e*Δ mitochondria in the presence or absence of a $\Delta\psi$. On treatment with proteinase K mitochondria were solubilized in digitonin-containing buffer and analyzed by BN-PAGE and digital autoradiography. (C) WT, *su e*Δ, and *su g*Δ mutant mitochondria were analyzed by BN-PAGE as described in A. (D) Mitochondria were incubated with radiolabeled Su k in the presence or absence of $\Delta\psi$. After treatment with proteinase K, samples were analyzed by SDS-PAGE and digital autoradiography. (E) Mitochondria were treated with 0.1 M sodium carbonate, pH 10.8. 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. M, monomeric F_1F_o-ATP synthase; M′, primed monomer; D′ and D, dimeric forms of F_1F_o-ATP synthase.

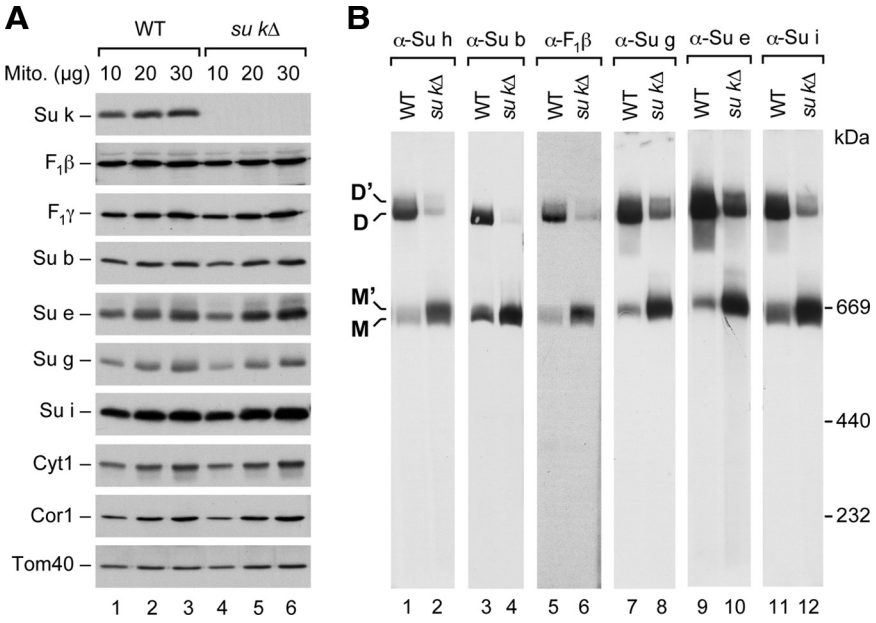

Figure 2. Su k is required for the formation of stable ATP synthase dimers. (A) Analysis of steady-state protein levels of wild-type (WT) and *su k*Δ mitochondria. Ten, 20, and 30 μg of mitochondrial proteins were analyzed by SDS-PAGE and Western blotting. (B) Isolated wild-type (WT) and *su k*Δ mitochondria were solubilized in buffer containing 1% digitonin and analyzed by BN-PAGE and immunoblotting with the indicated antisera. M, monomeric F_1F_o-ATP synthase; M′, primed monomer; D′ and D, dimeric forms of F_1F_o-ATP synthase.

et al., 1992; van der Laan *et al*., 2007). However, Su k is a very small protein (7.5 kDa), and we did not observe proteolytic processing of the Su k precursor in our import assays (Figure 1D). We therefore examined the membrane association of Su k by carbonate extraction. On incubation of mitochondria at pH 10.8 and subsequent ultracentrifugation, soluble proteins, such as aconitase (Aco1), and peripheral membrane proteins, such as Tim44, were found in the supernatant, whereas integral membrane proteins, such as Tim22, remained in the pellet fraction (Figure 1E). Su k is found in both pellet and supernatant fractions (Figure 1E) in similar amounts, indicating an unusual mode of association with the inner mitochondrial membrane, which may lead to the observed $\Delta\psi$ dependence of Su k import.

Su k Stabilizes Dimeric Forms of ATP Synthase

We then asked whether Su k is involved in the dimerization of F_1F_o-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 $F_1\beta$, $F_1\gamma$, and Su b; the small F_o-subunits Su e, Su g, and Su i; and the control proteins cytochrome c_1 (Cyt1), Cor1, and Tom40 were present in equal amounts in wild-type and *su k*Δ mitochondria (Figure 2A). ATP-dependent import of the radiolabeled $F_1\beta$ precursor protein into *su k*Δ 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 k*Δ mitochondria by high-resolution BN-PAGE and immunoblotting. Using antibodies against the peripheral stalk subunits Su h and Su b, the F_1-component $F_1\beta$ and the small F_o-subunits Su g, Su e, and Su i, we detected strongly reduced amounts of ATP synthase dimers in *su k*Δ 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 F_1F_o-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 k*Δ mitochondria and analyzed the assembly of these proteins with F_1F_o-ATP synthase. Incorporation of the central stalk subunit $F_1\gamma$ (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 $F_1\gamma$ and Su h with dimeric ATP synthase was observed in *su k*Δ mitochondria, reflecting the reduced steady-state levels of dimers (compare Figures 2B and 3, A and B). On import into *su k*Δ 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 k*Δ 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 F_1F_o-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 F_1F_o-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 k*Δ 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 k*Δ mitochondria assembly of Su h into dimers and oligomers of F_1F_o-ATP synthase

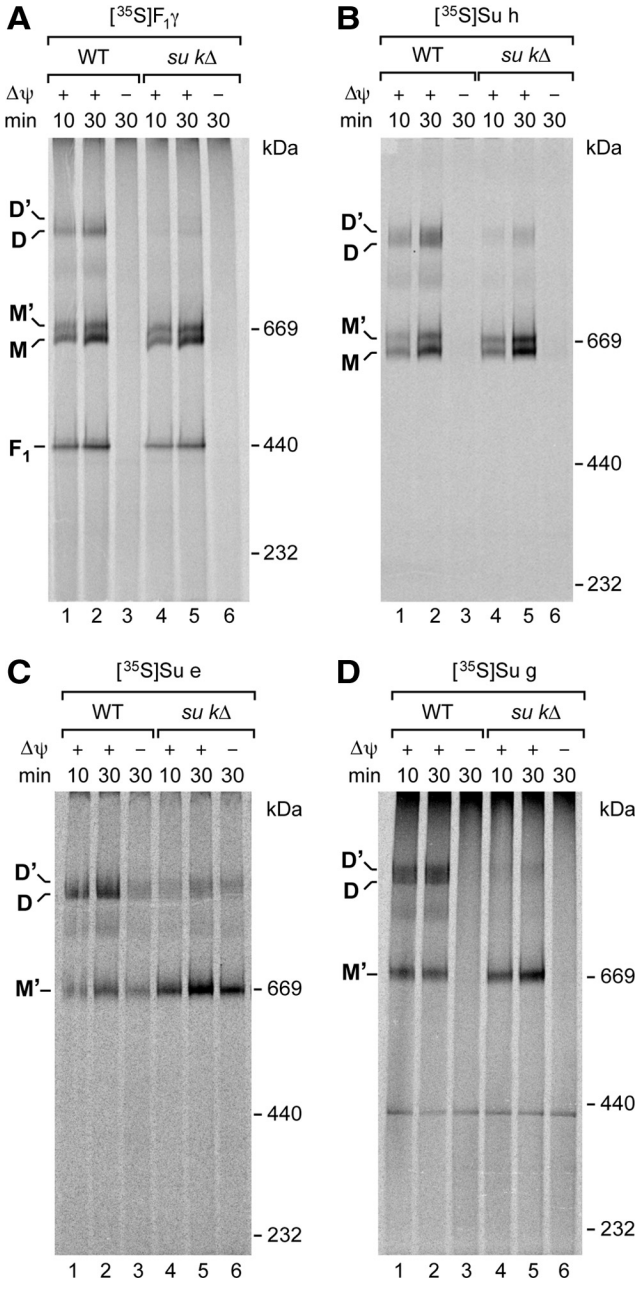

Figure 3. The primed monomer of F_1F_o-ATP synthase accumulates in the absence of Su k. (A) Import of radiolabeled $F_1\gamma$ into wild-type and *su k*Δ mitochondria in the presence or absence of a $\Delta\psi$. After import reactions mitochondria were treated with proteinase K and solubilized in digitonin buffer. Protein extracts were separated on 4–8% polyacrylamide blue native gels and analyzed by digital autoradiography. (B–D) Import of Su h, Su e, and Su g was performed and analyzed as described in A. Note that the presence of $\Delta\psi$ is not essential for import and assembly of Su e (Wagner *et al.*, 2009). M, monomeric F_1F_o-ATP synthase; M′, primed monomer; D′ and D, dimeric forms of F_1F_o-ATP synthase; F_1, free F_1-subcomplex.

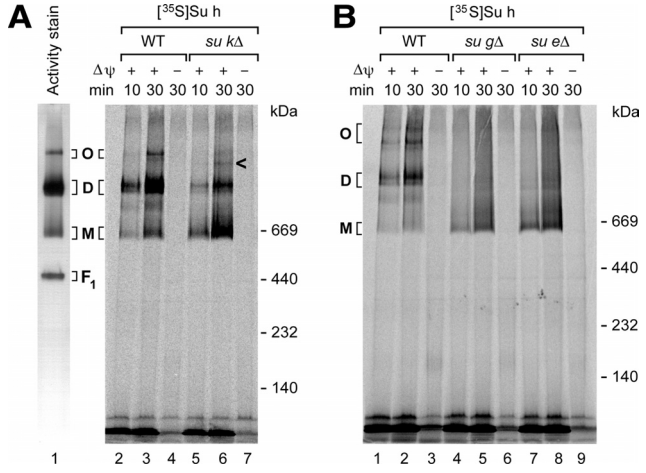

Figure 4. Higher oligomeric states of ATP synthase depend on Su k. (A) Mitochondria were solubilized in 0.35% digitonin and subjected to BN-PAGE by using 3–13% polyacrylamide gels. Activity staining of F_1F_o-ATP synthase complexes (lane 1) was carried out as described in *Materials and Methods*. ^{35}S-Labeled Su h was imported into wild-type (WT) and *su k*Δ mitochondria in the presence or absence of a $\Delta\psi$. After solubilization of mitochondria in digitonin buffer (0.35%) and BN-PAGE, gels were analyzed by digital autoradiography. (B) Import of radiolabeled Su h into WT, *su g*Δ, and *su e*Δ mitochondria was performed and analyzed as outlined in A. M, ATP synthase monomers; D, ATP synthase dimers; O, oligomeric forms of ATP synthase; F_1, F_1-subcomplex; arrowhead, partially fragmented oligomer. The gel system used here to detect larger oligomeric forms does not clearly separate the distinct forms of ATP synthase monomers and dimers.

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*Δ 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*Δ and *su e*Δ 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 F_1F_o-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 F_1F_o-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 F_1F_o-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*Δ) 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 F_1-part of ATP synthase ($F_1\beta$ and $F_1\gamma$), components of the F_o-sector, such as the catalytic subunit Su 6; the small

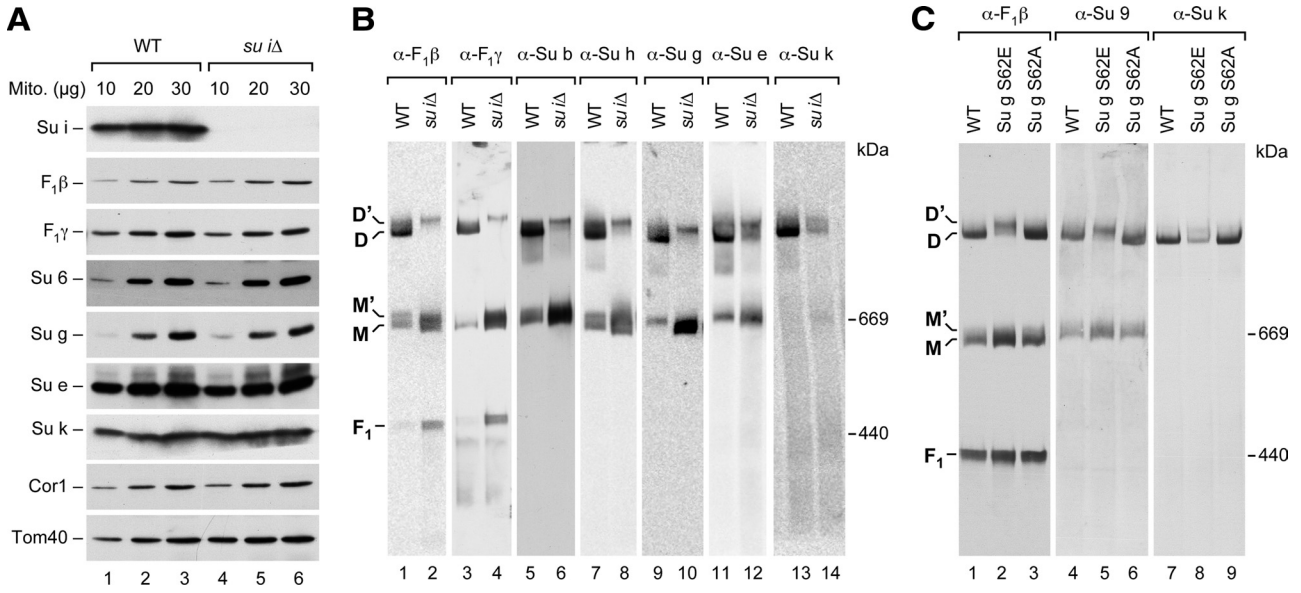

Figure 5. Su i is required for the formation of a distinct F_1F_o-ATP synthase dimer form. (A) Analysis of steady-state protein levels in wild-type (WT) and *su i*Δ mitochondria. Ten, 20, and 30 μg of mitochondrial proteins were analyzed by SDS-PAGE and immunoblotting. (B) Digitonin-solubilized wild-type (WT) and *su i*Δ mitochondria were subjected to high-resolution BN-PAGE and analyzed by Western blotting with the indicated antisera. (C) Isolated WT, Su g S62E, and 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 $F_1\beta$, Su 9 and Su k. M, monomeric ATP synthase; M′, primed monomer; D′ and D, dimeric forms of F_1F_o-ATP synthase; F_1, detached F_1-subcomplex.

subunits Su g, Su e, and Su k; and control proteins, such as the cytochrome bc_1 complex subunit Cor1 and the outer membrane protein Tom40, were present in *su i*Δ mitochondria in equal amounts compared with wild-type mitochondria (Figure 5A). Both the ATP-dependent import of the precursor of $F_1\beta$ (Supplemental Figure 1B, lanes 1–10) and the membrane potential across the inner mitochondrial membrane (Supplemental Figure 1C) were similar in wild-type and *su i*Δ mitochondria, indicating that the overall energy state was comparable. We solubilized wild-type and *su i*Δ mitochondria in buffer containing 1% digitonin and analyzed protein complexes by high-resolution BN-PAGE. Lack of Su i had profound effects on the different F_1F_o-ATP synthase complex forms. Using antibodies against $F_1\beta$, the central stalk component $F_1\gamma$, the peripheral stalk subunits Su b and Su h or the small peripheral F_o-subunits Su g and Su e, we observed much less dimeric ATP synthase in *su i*Δ 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 *su i*Δ mitochondria compared with the wild-type situation (Figure 5B, lanes 1–12). Thus, in the absence of Su i the dimer D′ is still formed, whereas generation of D is inhibited. As shown in Figure 1A, 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 Su k bound to F_1F_o-ATP synthase was strongly

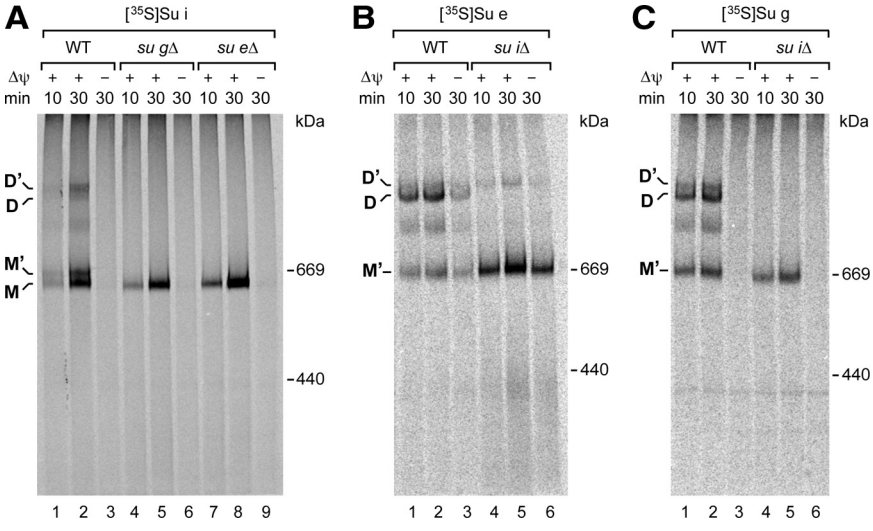

Figure 6. Independent assembly of Su i and Su e/g with monomeric ATP synthase. (A) Wild-type (WT), *su g*Δ, and *su e*Δ mitochondria were incubated with ^{35}S-labeled Su i in the presence or absence of a $\Delta\psi$. Subsequent to proteinase K treatment, mitochondria were solubilized in digitonin-containing buffer. Samples were applied to a 4–8% blue native gel and protein complexes were visualized by digital autoradiography. (B and C) Radiolabeled Su e and Su g were imported into WT and *su i*Δ mitochondria. Samples were analyzed as described in A. M, monomeric ATP synthase; M′, primed monomer; D′ and D, dimeric forms of F_1F_o-ATP synthase.

reduced in *su i*Δ 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 i*Δ mitochondria that was observed with antibodies against $F_1\beta$ and $F_1\gamma$ (Figure 5B, lanes 1–4) but not with antibodies against the F_o-subunits Su b and Su h (Figure 5B, lanes 5–8). Therefore, this complex species probably represents a detached F_1-subcomplex indicating that the interaction between F_1- and F_o-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 F_1F_o-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 F_1-protein $F_1\beta$ and the F_o-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 i*Δ 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 g*Δ and *su e*Δ 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 i*Δ mutant mitochondria. Su e and Su g assembled with monomeric F_1F_o-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 i*Δ 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-

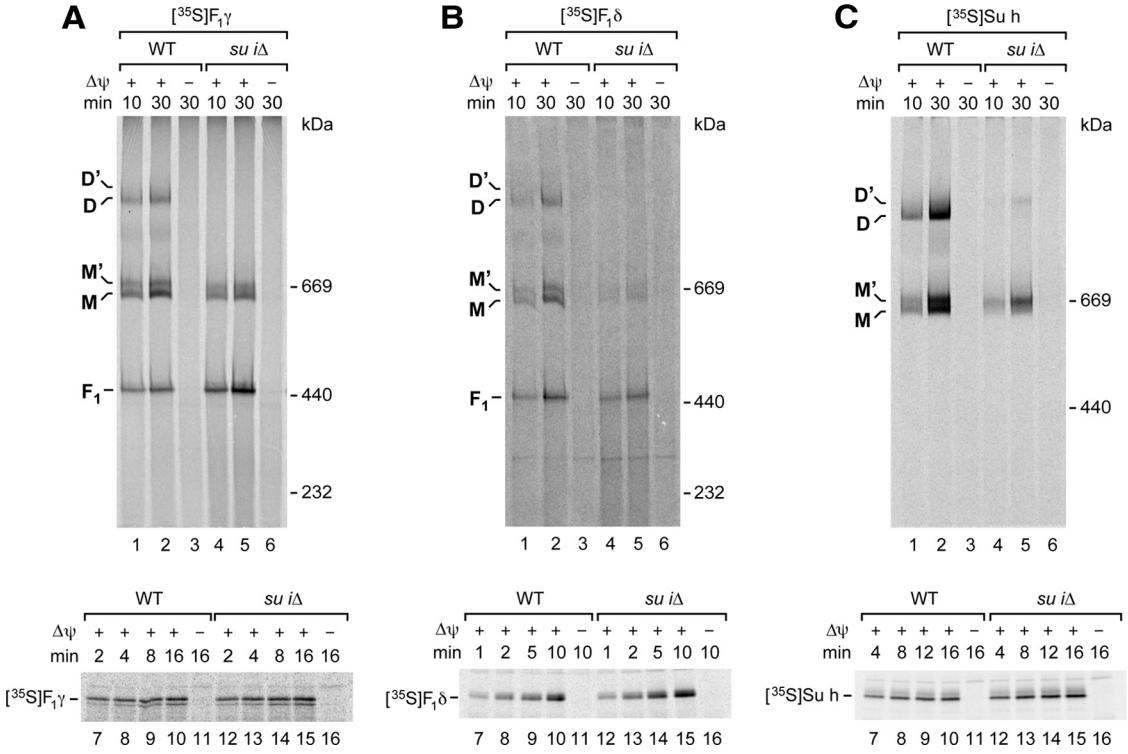

Figure 7. Su i facilitates the incorporation of imported subunits into central and peripheral stalk regions. ^{35}S-Labeled $F_1\gamma$ (A), $F_1\delta$ (B), and Su h (C) were imported into wild-type (WT) and *su i*Δ mitochondria in the presence or absence of a $\Delta\psi$. On treatment with proteinase 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 F_1F_o-ATP synthase; F_1, F_1-subcomplex.

bly of Su e and Su g with F_1F_o-ATP synthase are independent events.

Su i Facilitates the Assembly of Imported Subunits into Monomeric F_1F_o-ATP Synthase

We then asked how the absence of Su i affects the assembly of other newly imported subunits into the different F_1F_o-ATP synthase forms. We synthesized the radiolabeled precursors of several subunits of F_1F_o-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 $F_1\gamma$ (Figure 7A, lanes 4–6) and $F_1\delta$ (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, $F_1\gamma$ 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 $F_1\delta$ (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 F_1F_o-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 F_1F_o-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 F_1F_o-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

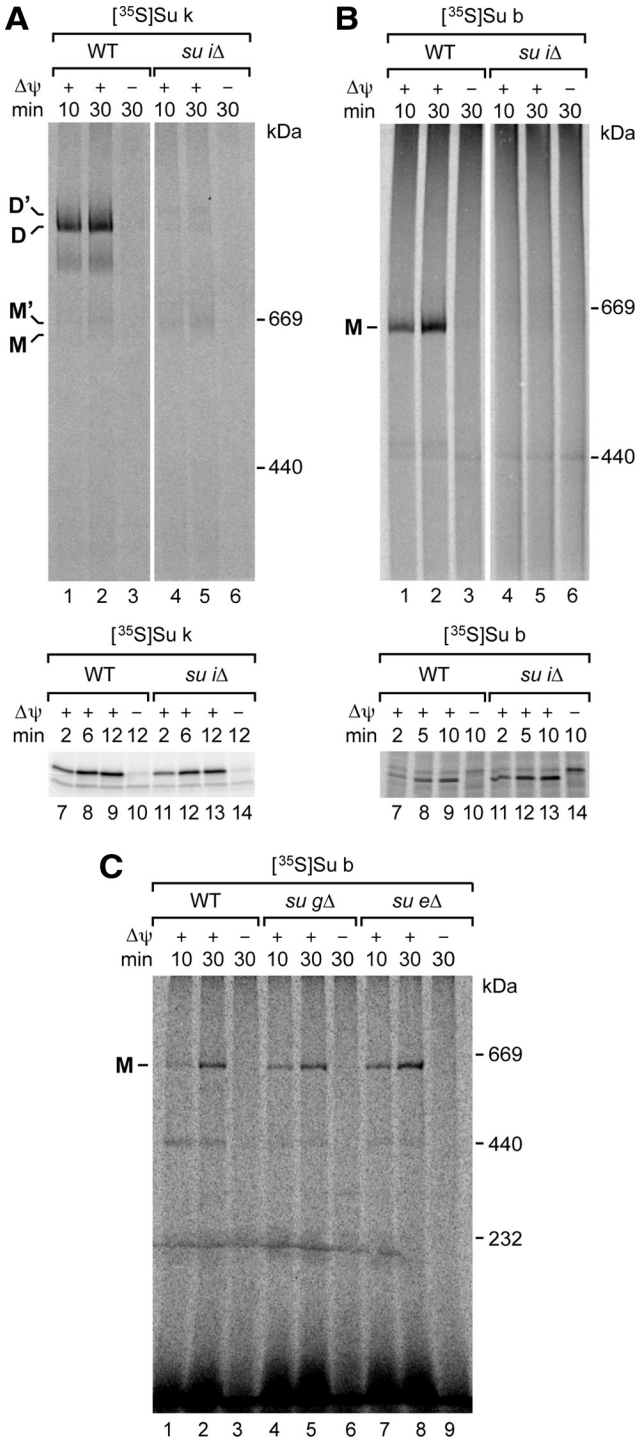

Figure 8. Su i is required for the assembly of newly synthesized Su k and Su b with F_1F_o-ATP synthase. (A) Wild-type (WT) and *su i*Δ mutant mitochondria were incubated with the radiolabeled precursor of Su k in the presence or absence of a $\Delta\psi$. After proteinase K-treatment, mitochondria were 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 ^{35}S-labeled Su b was carried out and analyzed as described in A. (C) ^{35}S-Labeled Su b was imported into WT, *su g*Δ, and *su e*Δ mitochondria and analyzed by BN-PAGE and digital autoradiography as described in A. M, monomeric F_1F_o-ATP synthase; M′, primed monomer; D′ and D, dimeric forms of ATP synthase.

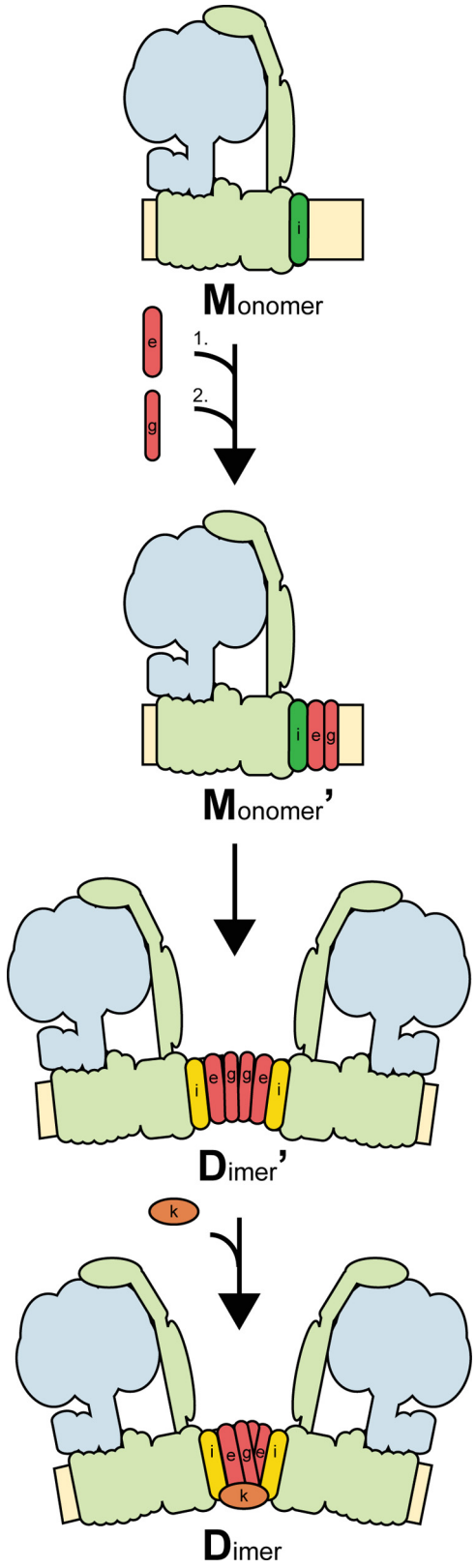

Figure 9. Model for stepwise assembly of dimeric F_1F_o-ATP synthase in mitochondria. Sequential association of Su e and Su g with monomeric ATP synthase (M) leads to the formation of a primed monomer (M′). An intermediate dimeric form (D′) assembles from these primed monomers. Su i drives the conversion 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 incorporation of new subunits into F_1F_o-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.

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_1F_o-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_1F_o-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_1F_o-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

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_1F_o-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_1F_o-ATP synthase. Together, these data suggest that D′ is an intermediate form of the dimeric F_1F_o-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 kΔ* 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_1F_o-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_1F_o-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_o-subunits.

Together, our study indicates that the biogenesis of stable F_1F_o-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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