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Formation of the Yeast F_1F_0-ATP Synthase Dimeric Complex Does Not Require the ATPase Inhibitor Protein, Inh1*

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The yeast F_1F_0-ATP synthase forms dimeric complexes in the mitochondrial inner membrane and in a manner that is supported by the F_0-sector subunits, Su e and Su g. Furthermore, it has recently been demonstrated that the binding of the F_1F_0-ATPase natural inhibitor protein to purified bovine F_1-sectors can promote their dimerization in solution (Çabezon, E., Arechaga, I., Jonathan P., Butler, G., and Walker J. E. (2000) *J. Biol. Chem.* 275, 28353–28355). It was unclear until now whether the binding of the inhibitor protein to the F_1 domains contributes to the process of F_1F_0-ATP synthase dimerization in intact mitochondria. Here we have directly addressed the involvement of the yeast inhibitor protein, Inh1, and its known accessory proteins, Stf1 and Stf2, in the formation of the yeast F_1F_0-ATP synthase dimer. Using mitochondria isolated from null mutants deficient in Inh1, Stf1, and Stf2, we demonstrate that formation of the F_1F_0-ATP synthase dimers is not adversely affected by the absence of these proteins. Furthermore, we demonstrate that the F_1F_0-ATPase monomers present in *su e* null mutant mitochondria can be as effectively inhibited by Inh1, as its dimeric counterpart in wild-type mitochondria. We conclude that dimerization of the F_1F_0-ATP synthase complexes involves a physical interaction of the membrane-embedded F_0 sectors from two monomeric complexes and in a manner that is independent of inhibitory activity of the Inh1 and accessory proteins.

Mitochondria, eukaryotic organelles, produce energy in the form of adenosine 5′-triphosphate (ATP) in a process termed oxidative phosphorylation (for recent reviews, see Refs. 1–3). Under conditions of high proton motive force ($\Delta\mu H^+$), the F_1F_0-ATP synthase complex catalyzes the formation of ATP from adenosine 5′-diphosphate (ADP) in a manner that is coupled to the transport of protons from the intermembrane space across the inner membrane to the matrix. A decrease in the $\Delta\mu H^+$ as a result of oxygen deprivation to the cell, or because of the presence of an uncoupler of oxidative phosphorylation can, however, cause a reversal of the action of the F_1F_0-ATP synthase, resulting in the hydrolysis of ATP to ADP and P_i. This hydrolytic activity of the F_1F_0-ATP synthase is regulated directly by the natural inhibitor protein, termed IF_1 in bovine and Inh1 in yeast. Under conditions of low $\Delta\mu H^+$, the IF_1 protein forms homodimers (its active state) and binds directly to the F_1-sector and by doing so promotes the inhibition of ATP hydrolysis and thereby preserves cellular ATP levels (4–7). Homodimerization of IF_1 is supported by a coiled-coil structural motif in the IF_1 protein and occurs under conditions of matrix acidification (4–7). IF_1 binds to the F_1-sector in a 1:1 stoichiometry (8). Using purified F_1-sectors, the active IF_1 dimer has been shown to be able to bind to two separate F_1 complexes concomitantly, thereby promoting their dimerization (6, 7). Although the IF_1 protein can promote dimerization of F_1 complexes in solution, it was not apparent until now whether the inhibitor protein may play a direct role in the formation of dimers of the membrane-bound F_1F_0-ATP synthase complex in mitochondria.

It has been recently demonstrated that the yeast mitochondrial F_1F_0-ATP synthase forms dimeric complexes in the inner membrane (9, 10). The dimeric ATP synthase complex was identified following mild detergent (digitonin) solubilization of mitochondrial membranes, followed by either size exclusion chromatography or by blue-native polyacrylamide gel electrophoresis (BN-PAGE)[1] (9, 10). The proximity between two F_1F_0-ATP synthase complexes was also independently shown by the ability to form a disulfide bridge between two subunits 4 proteins from neighboring ATP synthase complexes (11). Analysis of the subunit composition of the dimeric and monomeric complexes following BN-PAGE, by high-resolution two-dimensional gel electrophoresis resulted in the identification of dimer-specific subunits e and g (Su e and Su g, respectively) (10). Although not essential for the enzyme activity of the complex, Su e and Su g were shown to play an important role in the formation of the dimeric state of the F_1F_0-ATP synthase (10). BN-PAGE analysis indicated that formation of the dimeric form of the F_1F_0-ATP synthase complex was affected in mitochondria isolated from Su e and Su g yeast null mutants (Δ*su e* and Δ*su g*, respectively) (10). The dimeric state of the ATP synthase is present also in both bovine and human mitochondria, indicating this assembly state of the complex is not unique to yeast mitochondria (12, 13). Consistently, subunits Su e and Su g, required for the formation of the dimeric ATP synthase, are conserved throughout evolution, present in both fungal and mammalian mitochondria.

As integral inner membrane proteins, Su e and Su g are both subunits of the F_0-sector. The dimerization of the F_1F_0-ATP synthase complexes was thus proposed to involve a physical interaction of two membrane-embedded F_0-sectors from two monomeric F_1F_0-ATP synthase complexes (10). The recent ob-

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[1] The abbreviations used are: BN-PAGE, blue-native polyacrylamide gel electrophoresis; STF, stabilizing factor; Sfl2, stabilizing factor 2-like protein; MCS, multiple cloning site; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone.

servation that purified F_1-sectors can dimerize in solution upon binding of the natural inhibitor protein, however, raises an important question as to whether the inhibitor protein binding may play a role in the formation of the F_1F_0-ATP synthase dimers in the mitochondrion. Although dimerization of the F_1F_0-ATP synthase complex requires the presence of the F_0-sector Su e, it is plausible that the binding and inhibitory action of the IF_1/Inh1 protein may drive or support the dimerization of the F_1F_0-ATP synthase complex in the mitochondrial membrane. In this present study, we have directly addressed the role of the inhibitor protein, Inh1, in the formation of the F_1F_0-ATP synthase dimer in mitochondria isolated from the yeast *Saccharomyces cerevisiae*.

In yeast the inhibitory action of the Inh1 protein is enhanced by two stabilizing proteins, which are termed Stf1 and Stf2 (STF = stabilizing factors) (14–19). Stf1 shares sequence similarity with Inh1 (51% identity) and like Inh1, displays the potential to form a coiled-coil structure. The amino acid sequence of Stf2 on the other hand is unrelated to the Inh1/Stf1 proteins and does not contain a predicted coiled-coil motif. A data base search, however, indicated the presence of a homolog of Stf2 in yeast, which is encoded by the gene *YLR327c*. The function of the *YLR327c* gene product is unknown to date. The predicted protein encoded by this open reading frame is 86 amino acid residues long (Stf2 is 84 residues long) and is referred to here as Sfl2, stabilizing factor 2-like protein 2. The amino acid sequence of Sfl2 displays 65% identity and 84% similarity with that of the Stf2 protein.

Here we have addressed the role of Inh1 and its accessory proteins Stf1 and Stf2 in the formation of the dimeric F_1F_0-ATP synthase complex. We demonstrate here that the assembly of F_1F_0-ATP synthase dimers is not adversely affected by the absence of the Inh1, Stf1, Stf2, or Sfl2 proteins. We propose therefore that dimerization of the mitochondrial F_1F_0-ATP synthase is primarily supported through membrane-embedded F_0-sector subunits Su e and Su g, and in a manner that is independent of the Inh1 and accessory proteins. Finally, we demonstrate that both the steady state levels of Inh1 and accessory proteins, and their capacity to inhibit the ATP hydrolysis activity of the F_1F_0-ATP synthase complex under low $\Delta\mu H^+$ conditions, are not affected in the Δ*su e* mitochondria. We conclude therefore that formation of the Su e-mediated F_1F_0-ATP synthase dimers is not required for the inhibition of the ATPase activity of the complex by the Inh1 inhibitor and accessory proteins.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions

Yeast strains used in this study were wild-type W303-1A (Mat**a**, *leu2, trp1, ura3, his3, ade2*) (20) and the *su e* null mutant, Δ*su e* (W303-1A, *leu2, trp1, ura3, ade2, TIM11*::*HIS3*) (10). The single gene deletion yeast strains, Δ*inh1*, Δ*stf1*, Δ*stf2*, Δ*sfl2*, were constructed in the W303-1A genetic background, as follows. The introduction of the kanamycin resistance gene (*KAN^r*) into the *INH1*, *STF1*, *STF2,* and *SFL2* (*YLR327c*) gene loci of wild-type cells, resulting in the complete or partial deletion of the respective open reading frames was performed. The *KAN^r* gene was amplified from plasmid pFA6a-KANMX6 (21) using the following forward and reverse primers, S1 and S2, specific for each target gene, as outlined below.

For the INH1 Gene, Δinh1::KAN^r(Δinh1)

S1: 5′-cgcattactacagcacacttttatacagttccacaatagaatatgcgtacgctgcaggtcgac-3′ (corresponds to nucleotides −42 to +3 of the *INH1* gene locus and 18 nucleotides in the multiple cloning site (MCS) of the pFA6a-KANMX6 from the 5′-flanking region of the *KAN^r* gene) and S2: 5′-tatatagtttttctgctgtttgactataaaagagtaagaatattcatcgatgaattcgagctcg-3′ (corresponds to nucleotides +295 to +339 of the *INH1* gene (+1 to +256 bp), which are located in the 3′ noncoding region of the *INH1* gene and 19 nucleotides of the MCS of the pFA6a-KANMX6 plasmid from the 3′-flanking region of the *KAN^r* gene).

For the STF1 Gene, Δstf1::KAN^r(Δstf1)

S1: 5′-gaagcactttcacccagtcatccaaccaaacacgctatcaatatgcgtacgctgcaggtcgac-3′ (corresponds to nucleotides −42 to +3 of the *STF1* gene locus and 18 nucleotides in the MCS of the pFA6a-KANMX6 plasmid, as described above), and S2: 5′-gatgttcatttcctaattttgatttggcatgattgcttctttctaatcgatgaattcgagctcg-3′ (corresponds to nucleotides +307 to +351 of the *STF1* gene (+1 to +309 bp) located in the 3′ region of the *STF1* locus and 19 nucleotides of the MCS of the pFA6a-KANMX6 plasmid, as described above).

For the STF2 Gene, Δstf2::KAN^r(Δstf2)

S1: 5′-caacagtaacaaaccgctcaagtgtacaaccaatcagaaaaaatgcgtacgctgcaggtcgac-3′ (corresponds to nucleotides −42 to +3 of the *STF2* gene locus and 18 nucleotides in the MCS of the pFA6a-KANMX6 plasmid, as described above), and S2: 5′-atcaatctcatcgcctggcttaccccaattacccttgccggaaccatcgatgaattcgagctcg-3′ (corresponds to nucleotides +106 to +150 of the STF2 gene (+1 to +255 bp), which are located in the open reading frame of the *STF2* locus and 19 nucleotides of the MCS of the pFA6a-KANMX6 plasmid, as described above).

For the SFL2 Gene, Δsfl2::KAN^r(Δsfl2)

S1: 5′-3′-acattaaagcacacaaatttctaacacaaacacaattcaaacatgcgtacgctgcaggtcgac (corresponds to nucleotides −42 to +3 of the *YLR327c* (*SFL2*) gene locus and 18 nucleotides in the MCS of the pFA6a-KANMX6 plasmid, as described above), and S2: 5′-ttagatgtggtattgttgcaaatcagaaagccttctttcattgttatcgatgaattcgagctcg-3′ (corresponds to nucleotides +216 to +261 of the *SFL2* gene (+1 to +261 bp), which are located in the open reading frame of the *SFL2* locus and 19 nucleotides of the MCS of the pFA6a-KANMX6 plasmid, as described above).

The resulting PCR products were transformed into the haploid yeast strain W303-1A (20) using the protocol described in Ref. 21, and kanamycin-resistant transformants were selected. Correct integration of the *KAN^r* gene into the *INH1*, *STF1*, *STF2,* and *SFL2* gene loci were confirmed by PCR analysis on isolated genomic DNA and using oligonucleotides, which primed upstream and downstream of the respective disrupted genes (results not shown).

Construction of the Double Gene Null Strains, Δinh1/Δstf1 and Δstf2/Δsfl2

For the Δinh1/Δstf1 Strain, Δinh1::KAN^r/Δstf1::HIS3—The *STF1* gene locus was deleted in the Δ*inh1*::KAN yeast strain, as follows. The *HIS3* gene was amplified from the pFA6a-HIS3MX6 plasmid (21) using the *STF1*-specific S1 and S2 primers (see above). The resulting PCR product was transformed into the Δ*inh1*::KAN yeast strain and *HIS3*-positive transformants were selected. Correct integration of the *HIS3* gene into the *STF1* gene locus was verified by PCR analysis of the isolated genomic DNA, as described above.

For the Δstf2/Δsfl2 Strain, Δstf2::HIS3/Δsfl2::KAN^r—The *STF2* gene locus was deleted in the Δ*sfl2*::KAN yeast strain, as follows. The *HIS3* gene was amplified from the pFA6a-HIS3MX6 plasmid using the STF2-specific S1 and S2 primers (see above). The resulting PCR product was transformed into the Δ*sfl2*::KAN yeast strain and *HIS3*-positive transformants were selected. Correct integration of the *HIS3* gene into the *STF2* gene locus was verified by PCR analysis of the isolated genomic DNA, as described above. Mitochondria were isolated from the resulting yeast strains, which had been grown in YP-Gal medium (2% galactose) supplemented with 0.5% lactate (22).

Isolation of Mitochondria for BN-PAGE Analysis and ATPase Measurements

The individual single null mutant and double null mutant strains were grown on YP-medium containing galactose. Yeast cells were harvested by centrifugation, washed, and then disrupted with glass beads, essentially as described previously (10). For BN-PAGE analysis the cells were disrupted in a sucrose, 6-aminohexanoic acid buffer (250 mM sucrose, 5 mM 6-aminohexanoic acid, and 10 mM Tris-HCl, pH 7.0). For the ATPase measurements, the cells were grown on YP-glycerol, 0.5% lactate medium and were disrupted in SH buffer (0.6 M sorbitol, 20 mM Hepes, pH 7.2). Following vortexing with the glass beads, the cell debris and glass beads were initially removed by low speed centrifugation. The mitochondrial membranes were then collected by centrifugation at 18,000 × *g* for 20 min at 4 °C and were stored at −80 °C in a sucrose-containing buffer.

ATP Hydrolysis Measurements

ATPase activity measurements at pH 6.0—Isolated mitochondria (100 μg of protein) were resuspended in 525 μl of ice-cold assay buffer

(0.2 M KCl, 3 mM $MgCl_2$, 20 mM Hepes, pH 6.0), supplemented with 5 mM ATP and incubated in the presence or absence of oligomycin (20 μM) for 1 min on ice. Antimycin A (11 μM) and CCCP (20 μM) were then added and samples were incubated on ice for a further 30 s. Samples were transferred to 30 °C (t = 0 min) and at 15-s intervals, 50-μl aliquots were removed, placed on ice, and further ATP hydrolysis activity was stopped by the addition of 170 μl of 3 M trichloroacetic acid. The samples were centrifuged for 10 min at 10,000 rpm at 4 °C, and 200 μl of the supernatant was added to a malachite green/ammonium molybdate solution for the determination of the phosphate produced, as previously described (23, 24).

ATPase Activity Measurements at pH 8.4—ATP hydrolysis activity of the F_1F_0-ATP synthase activity at pH 8.4 in energized mitochondria was determined essentially as described above, with the following exceptions: the assay buffer used was 0.2 M KCl, 3 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, and the mitochondrial membrane potential was not dissipated, as antimycin A and CCCP were omitted.

BN-PAGE

BN-PAGE analysis of digitonin-solubilized mitochondrial membranes (3 g of digitonin/g of mitochondrial protein) was performed essentially as described previously (10, 13). The effect of acidic pH and a low proton motive force (*i.e.* inhibitor protein binding conditions) on the dimeric state of the ATP synthase, in comparison to that in energized mitochondria at pH 8.4, was assessed by BN-PAGE as follows: mitochondria (wild-type or Δ*su e* null mutant, as indicated) were incubated either with ice-cold, pH 6.0, buffer (0.2 M NaCl, 3 mM $MgCl_2$, 20 mM Hepes, pH 6.0, 2 mM ATP) in the presence of antimycin A (11 μM) and CCCP (20 μM) or pH 8.4 buffer (0.2 M NaCl, 3 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.4, 2 mM ATP) for 30 s on ice. Mitochondria were then reisolated by centrifugation and were prepared for BN-PAGE following lysis in digitonin, as described above.

Miscellaneous

Protein determinations and SDS-PAGE were performed according to published methods (25, 26). The Western blot analysis and immune decoration was performed using available Su e antisera (10) and Inh1 and Stf1 antisera (kind gift from Professor Tadao Hashimoto, Muroran Institute of Technology, Japan).

RESULTS

Formation of the Dimeric F_1F_0-ATP Synthase Complex Does Not Require the Presence of Inh1 and Its Accessory Proteins Stf1 and Stf2—To investigate the possible involvement of Inh1 and accessory proteins Stf1 and Stf2 in the dimerization of the F_1F_0-ATP synthase, we constructed single gene knock-out yeast strains, deficient in the gene encoding Inh1, Stf1, Stf2, or the Stf2 putative homolog, Sfl2 (encoded by the *YLR327c* gene) (see "Experimental Procedures" for details). The single gene deletions were performed in the haploid yeast strain W303-1A, which is the same genetic background as our existing Δ*su e* strain (10).

Mitochondria were initially isolated from the resulting four individual deletion yeast strains Δ*inh1*, Δ*stf1*, Δ*stf2,* and Δ*sfl2*, which had been grown on galactose-containing medium. Mitochondrial membrane proteins were solubilized with the detergent digitonin and the dimeric state of the F_1F_0-ATP synthase was then directly analyzed by BN-PAGE (Fig. 1). The isogenic wild type was analyzed in parallel, where both dimeric and monomeric forms of the ATP synthase were observed, as previously described (10). The dimeric ATP synthase complex was also observed in mitochondria isolated from each of the Δ*inh1*, Δ*stf1*, Δ*stf2*, and Δ*sfl2* strains. The ratio of dimeric to monomeric complex in these mutant mitochondrial types appeared unaltered when compared with wild-type mitochondria. We conclude therefore that the Inh1, Stf1, Stf2, and Sfl2 proteins alone do not play an essential role in the formation of the dimeric ATP synthase complex in yeast mitochondria.

To exclude the possibility of functional redundancy between homologous proteins Inh1 and Stf1 and between Stf2 and Sfl2, double gene deletion yeast strains, null for both homologous genes, the Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2* strains, respectively, were also constructed. Using digitonin, the membrane protein complexes were solubilized from the mitochondria isolated from the Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2* yeast strains. Both dimeric and monomeric forms of the F_1F_0-ATPase were observed to be present in both the Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2* mitochondria (Fig. 1). Furthermore, the SDS-PAGE second dimension resolution of the complexes indicated that the subunit composition of the monomeric and dimeric forms of the F_1F_0-ATPase were similar in mitochondria isolated from the Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2* and the isogenic wild-type strains (Fig. 2).

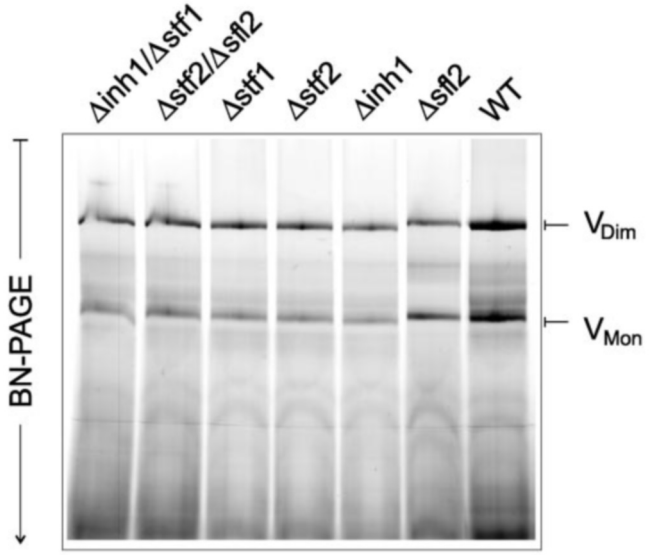

FIG. 1. **BN-PAGE analysis of the F_1F_0-ATP synthase.** The ATP synthase was solubilized from mitochondria isolated from the Δ*inh1*, Δ*stf1*, Δ*stf2*, Δ*sfl2* single gene deletion yeast strains and also from the Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2* mutant strains. Solubilization of membrane proteins was performed using a digitonin:protein ratio of 3.0 g/g. Samples were analyzed by BN-PAGE. The dimeric (V_{Dim}) and monomeric (V_{Mon}) forms of the ATP synthase are indicated.

On the basis of these data, we conclude that the formation of the dimeric F_1F_0-ATPase complex in yeast mitochondria does not require the presence of the Inh1 protein and its known accessory proteins Stf1 and Stf2, or the putative homolog of the Stf2 protein, termed Sfl2.

Inh1 and Its Accessory Proteins Do Not Display an Interdependence with Su e for Their Stable Expression—We next addressed whether Su e and Inh1 together with its accessory proteins display an interdependence on each other for their stable expression (Fig. 3). Mitochondria were isolated from the four individual mutant strains, Δ*inh1*, Δ*stf1*, Δ*stf2,* and Δ*sfl2,* and were analyzed by SDS-PAGE and Western blotting, together with mitochondria isolated from the corresponding wild-type strain. The levels of Su e were analyzed in these mutant mitochondria and were compared with a control mitochondrial protein, Tim23. Deletion of the genes encoding Inh1 or its accessory proteins had no appreciable effect on the steady state levels of Su e (Fig. 3*A*).

To assess the potential functional redundancy between Inh1 and its homolog Stf1, and between Stf2 and its putative homolog Sfl2, the steady state levels of Su e were analyzed in the mitochondria isolated from the double null mutant strains, Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2,* respectively. Western blot analysis using Su e-specific antisera confirmed that no significant alteration in the levels of Su e was observed in both the Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2* mitochondria, as compared with wild type (Fig. 3*B*).

We conclude that the presence of the Inh1 and accessory proteins does not appear to influence the steady state levels of

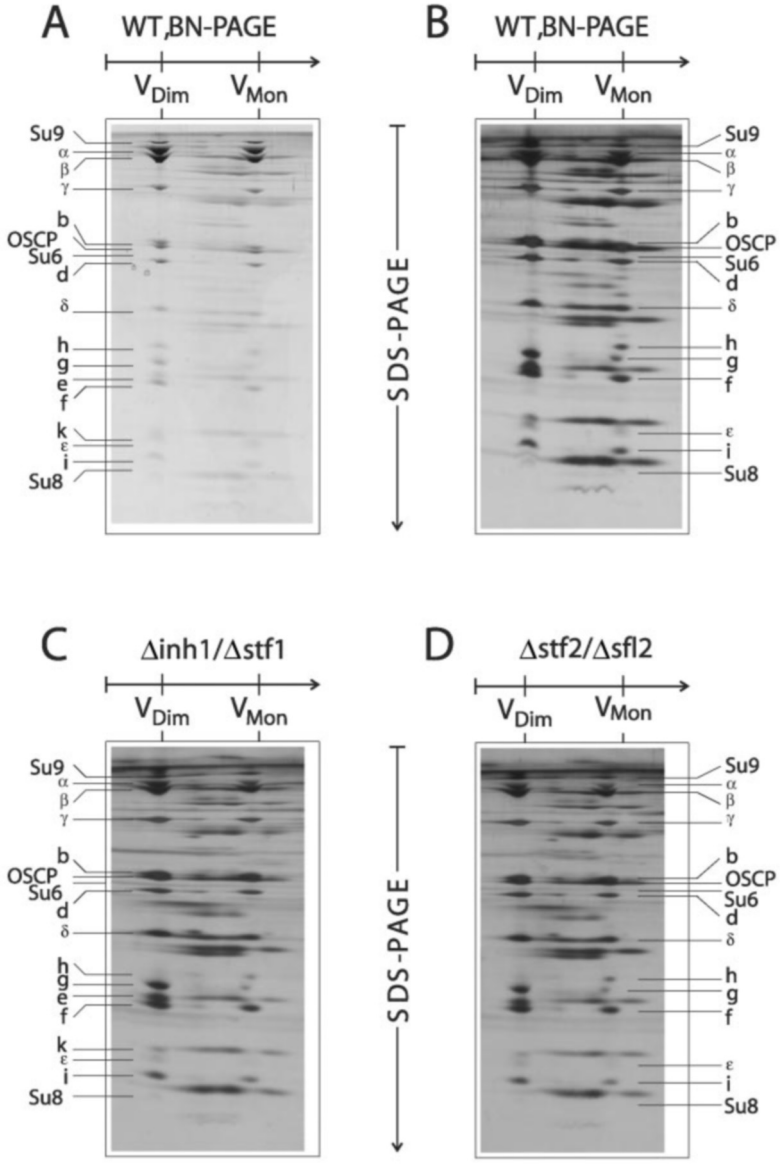

FIG. 2. **Polypeptide composition of the dimeric and monomeric ATP synthase from the Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2* mutant mitochondria.** The dimeric and monomeric forms of the F_1F_0-ATP synthase complex from wild-type (*panels A* and *B*) and the Δ*inh1*/Δ*stf1* (*panel C*) and Δ*stf2*/Δ*sfl2* (*panel D*) resolved by BN-PAGE were analyzed in a second dimension by a SDS-urea-PAGE. The resulting gels were either stained with Coomassie (*panel A*) or were silver stained (*panels B–D*). The positions of the dimeric (V_{Dim}) and monomeric (V_{Mon}) forms of the ATP synthase following BN-PAGE are indicated.

the dimer-specific subunit, Su e. These results are consistent with the observation that the formation of the dimeric form of the ATP synthase does not appear to be adversely affected in the *inh1* or accessory protein mutant mitochondria.

The influence of the dimer-specific subunit Su e on the stability of the Inh1 and Stf1 proteins was next investigated. Mitochondria were isolated from the Δ*su e* null mutant yeast strain and analyzed by SDS-PAGE and Western blotting (Fig. 3*C*). The levels of Inh1 and Stf1 in the mutant mitochondria were analyzed using subunit-specific antisera. The presence of the Su e was observed not to be required for the stable expression of Inh1 or Stf1, as the levels of these proteins in the Δ*su e* mitochondria were very similar to those in the wild-type control mitochondria. Note, the steady state levels of Stf2 and Sfl2 proteins in the Δ*su e* mitochondria have not been determined, as we do not have specific antisera available for these proteins yet.

As the assembly of the dimeric form of the ATP synthase is defective in the Δ*su e* mitochondria, we conclude that the stable expression of Inh1 and Stf1 does not require the presence of the assembled dimeric F_1F_0-ATP synthase. Taken together, the dimer-specific subunit Su e, and the inhibitor protein Inh1 and its accessory proteins do not display an interdependence on each other for their stable expression. These observations are consistent with those presented previously, where the formation of the dimeric ATP synthase, a process required for the stable expression of Su e, does not require the presence of the Inh1 or its accessory proteins, Stf1, Stf2, and its homolog, Sfl2.

Dimerization of the ATP Synthase Mediated by Su e, Is Not Required for the Inhibition of the ATPase Activity by Inh1—We next addressed whether formation of Su e-mediated F_1F_0 dimers in yeast was necessary for the ability of the Inh1 and accessory proteins to effectively inhibit the ATP hydrolysis activity of the F_1F_0-ATPase complex under the adverse conditions of low proton motive force ($\Delta\mu H^+$). Mitochondria were isolated from the Δ*su e* null mutant and also the Δ*inh*1 and Δ*inh1*/Δ*stf1* null mutant strains, and ATP hydrolysis catalyzed by oligomycin-sensitive F_1F_0-ATPase was measured at pH 6.0, following dissipation of the mitochondrial membrane potential (Fig. 4). As previously reported (18, 19), conditions of low $\Delta\mu H^+$, *i.e.* following the addition of an uncoupler such as CCCP, induces the ATP hydrolyzing activity of the F_1F_0-ATPase complex in mitochondria isolated from the inhibitor-deficient (Δ*inh1*) yeast cells (Fig. 4). Furthermore, consistent with previously published results (18, 19), the induction of ATP hydrolysis activity was more pronounced in the Δ*inh1*/Δ*stf1*

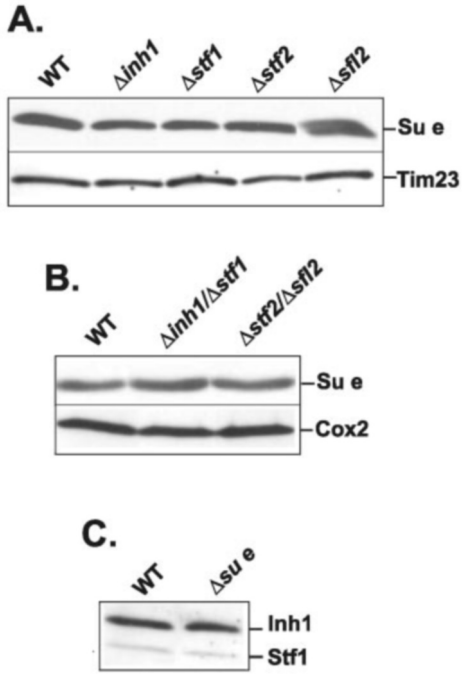

FIG. 3. **Steady state levels of the Su e, Inh1, and Stf1 proteins.** Mitochondria (50 μg of protein) isolated from the Δ*inh1*, Δ*stf1*, Δ*stf2*, and Δ*sfl2* single gene deletion yeast strains (*panel A*), the Δ*inh1*/Δ*stf1* and Δ*stf2*/Δ*sfl2* double null mutant strains (*panel B*), and the Δ*su e* null strain (*panel C*), together with mitochondria isolated from the corresponding isogenic wild-type (*WT*) strain were subjected to SDS-PAGE and Western blotting. The blots were decorated with antibodies specific for Su e, Inh1 and Stf1, and Tim23 or Cox2, as indicated.

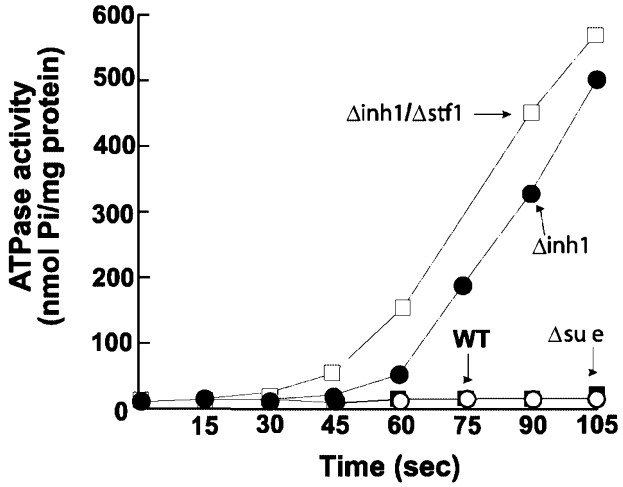

FIG. 4. **Measurement of F_1F_0-ATPase activities under low $\Delta\mu H^+$ and acidic pH conditions.** Oligomycin-sensitive ATP hydrolysis was measured in isolated mitochondria at pH 6.0, following dissipation of the membrane potential by the addition of antimycin A and CCCP, as described under "Experimental Procedures." Measurements were performed with isolated wild-type (*WT*, ○), Δ*su e* (■), Δ*inh1* (●), and Δ*inh1*/Δ*stf1* (□) mitochondria, as indicated.

mitochondria, where both Inh1 and Stf1 proteins are absent. In contrast, induction of ATP hydrolysis in this manner was not observed in wild-type mitochondria under these low $\Delta\mu H^+$ and matrix acidification conditions, because of the presence of the inhibitor protein, Inh1, which binds to and inhibits the F_1F_0-ATPase complex (Fig. 4). As was observed in wild-type mitochondria, the F_1F_0-ATP hydrolysis activity was not induced in the Δ*su e* mitochondria, following dissipation of the membrane potential. To control the F_1F_0-ATPase complex was indeed active in our Δ*su e* and wild-type mitochondrial preparations, oligomycin-sensitive ATP hydrolysis activities were determined in energized mitochondria from each strain at a pH of 8.4. The results obtained indicated that the mitochondria isolated from the wild type, *su e* null and *inh1*/*stf1* null mutant strains all had similar levels of oligomycin-sensitive F_1F_0-ATPase activity, ranging between 800 and 966 nmol of Pi/min/mg of protein.

In summary, under conditions of low $\Delta\mu H^+$ and an acidic milieu, it appears that F_1F_0-ATPase monomers in the Δ*su e* mitochondria can be as effectively inhibited by Inh1 as the dimeric complex in the wild-type mitochondria. As shown earlier, the steady state levels of Inh1 and Stf1 proteins appeared to be very similar between the Δ*su e* mitochondria and the wild-type mitochondria. Thus, the observed efficient inhibition of the monomeric ATP synthase in the Δ*su e* mitochondria by Inh1 does not appear to be because of a compensatory effect of overproduction of Inh1 relative to the wild type control.

Association of the Inh1 Protein with the Monomeric F_1F_0-ATP Synthase—The observed inhibition of the F_1-ATPase activity in the Δ*su e* mitochondria under conditions of acidic pH and low proton motive force, would indicate the ability of the Inh1 protein to effectively bind and inhibit the F_1F_0-ATP synthase monomer. The inhibitory action of the Inh1 protein in the Δ*su e* mitochondria does not appear to promote the stable dimerization of the monomeric F_1F_0-ATP synthase in the absence of subunit e, however (Fig. 5*A*). Preincubation of Δ*su e* mitochondria at pH 6.0 combined with dissipation of the membrane potential with CCCP and antimycin A, *i.e.* conditions that promote Inh1 binding, did not support dimer formation in the Δ*su e* mitochondria (Fig. 5*A*). This result would suggest that the inhibitor protein can effectively inhibit the ATPase activity of the monomeric F_1F_0 complex without promoting its dimerization. Consistently, analysis of the subunit composition of the ATP synthase complexes from wild-type mitochondria indicated the presence of the inhibitor protein Inh1 protein associated with both the dimeric and monomeric forms of the F_1F_0-ATP synthase complex (Fig. 5*B*). We conclude therefore, that the binding of the Inh1 protein to the monomeric F_1F_0-ATP synthase can occur and that Inh1 binding does not automatically promote ATP synthase dimer formation in intact mitochondria.

DISCUSSION

We have reported previously that the yeast F_1F_0-ATP synthase can be isolated as a dimeric complex from the mitochondrial inner membrane (10). Isolation of the dimeric complex was achieved following detergent lysis of the mitochondrial membranes using low detergent to protein ratios (9, 10). The nonessential ATP synthase subunits, Su e and Su g, were shown to play a critical role in formation of a stable F_1F_0-ATP synthase dimer (10). As both of these subunits are integral inner membrane proteins, this led us to propose the model that the formation of the dimeric F_1F_0-ATP synthase required a direct interaction between membrane-embedded F_0 segments (10). Recently, however, the observation that solubilized bovine F_1 domains can dimerize upon binding of the natural inhibitor protein, IF_1 (6, 7), has raised the question as to whether the dimerization of the intact F_1F_0 complex in the mitochondrial membrane system may be modulated by the binding and activity of IF_1.

In this present study we have directly addressed the role of Inh1, the yeast homolog of IF_1, in the formation of the dimeric ATP synthase in yeast mitochondria. We have also analyzed the possible roles of the Inh1 accessory proteins, Stf1 and Stf2, together with the putative Stf2 homolog, termed Sfl2. On the basis of our observations we argue that in yeast mitochondria,

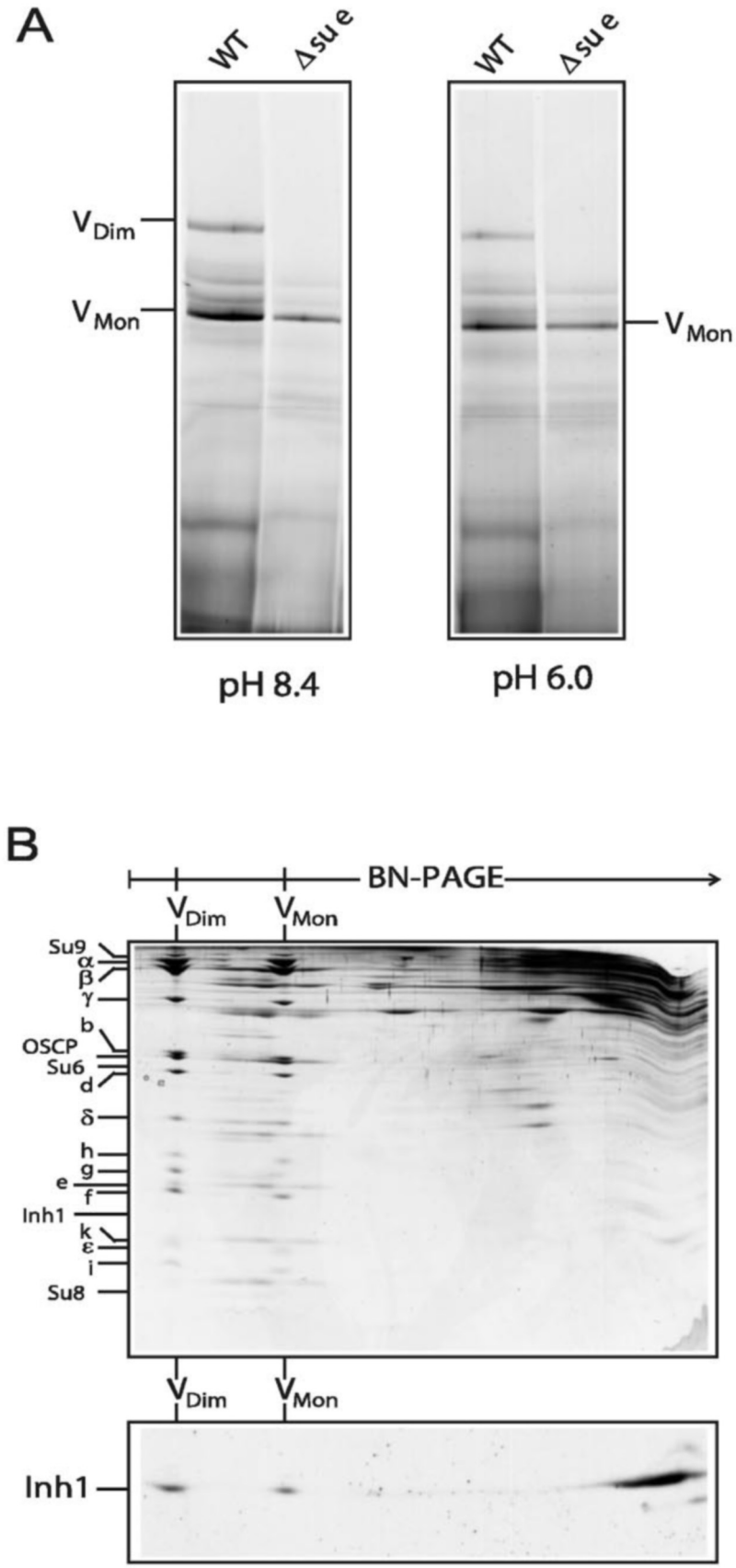

FIG. 5. **Association of Inh1 with both dimeric and monomeric forms of the F_1F_0-ATP synthase.** *A*, mitochondria isolated from wild-type or the Δ*su e* mutant, were incubated either at pH 8.4 in the presence of an energized membrane (pH 8.4), or at pH 6.0 in the presence of antimycin A and CCCP (pH 6.0), as described under "Experimental Procedures." Mitochondria were reisolated and were solubilized with digitonin and subjected to BN-PAGE analysis. *B*, the dimeric and monomeric forms of the F_1F_0-ATP synthase complex from wild-type mitochondria resolved by BN-PAGE were analyzed in a second dimension by a SDS-urea-PAGE and then Coomassie stained (*upper panel*). A duplicate gel was subjected to Western blotting and then was immune decorated with antibodies specific for the Inh1 protein (*lower panel*). The positions of the dimeric (V_{Dim}) and monomeric (V_{Mon}) forms of the ATP synthase following the BN-PAGE, and the presence of the Inh1 protein in both forms of the ATP synthase complex, are indicated. Note, the majority of the Inh1 protein was not associated with the ATP synthase, and was detected in the running front of the BN-PAGE gel.

the presence of the Inh1 or accessory proteins do not play an essential role in the formation of the dimeric ATP synthase complex. First, disruption of the gene encoding Inh1 alone, or in combination with the gene encoding its homolog, Stf1, had no adverse affect on the stability or subunit composition of the ATP synthase dimer. Likewise, the formation and subunit composition of the ATP synthase was also unaffected in the single or double null mutants of the other Inh1 accessory protein,

Stf2, and its putative homolog Sfl2. Second, if binding of the IF_1/Inh1 protein to the F_1-sector did indeed promote dimerization of the F_1F_0-ATP synthase complexes one may anticipate the dimer form to be a dynamic structure in mitochondrial inner membrane. According to this model, the monomeric ATP synthase would be recruited into a dimeric complex upon the concomitant binding of an active IF_1/Inh1 dimer to two neighboring F_1 domains. We have used the technique of BN-PAGE here to analyze the ratio of dimeric to monomeric ATP synthase in isolated wild-type mitochondria under conditions of low $\Delta\mu H^+$ or acidic pH, *i.e.* conditions that should promote IF_1/Inh1 binding, and observed no difference, relative to control mitochondria. Furthermore, dimerization of the monomeric ATP synthase in Δ*su e* mitochondria was not observed following incubation under these conditions optimal for Inh1 binding and inhibition.

Taken together, our current data would support a model that formation of the dimeric ATP synthase is not a dynamic process, which occurs in response to the binding of the Inh1 protein. Although the addition of active IF_1 dimers to purified F_1-sectors could promote their dimerization in solution at the ratio of IF_1:F_1-sector used (6, 7), we have not observed Inh1-mediated dimerization of F_1 complexes in intact mitochondria. We show here that the Inh1 protein can be associated with both dimeric and monomeric forms of the F_1F_0-ATP synthase. On the basis of our observations reported here, we conclude that the binding of IF_1/Inh1 to F_1-sectors in intact mitochondria does not play an essential role in the formation of the F_1F_0-ATP synthase dimers. Rather, as previously proposed (10), dimerization entails the association of the membrane-embedded F_0-ATP synthase subunits, in particular Su e. Furthermore, we propose dimerization of the ATP synthase involves formation of Su e-Su e homodimers, between two neighboring F_0 complexes. Sequence analysis of known Su e proteins show they share a conserved coiled-coil motif, the basis often for homodimerization. Indeed, preliminary evidence for the dimerization of Su e in bovine mitochondria was presented earlier (27). In addition, we have recently been able to directly show that yeast Su e forms homodimers in the mitochondrial inner membrane.[2]

What is the function of the dimeric form of the F_1F_0-ATP synthase complex? Although required for the formation of the dimeric complex, Su e is not an essential subunit for the enzymatic activity of the ATP synthase complex (10). The amino acid sequence of Su e is strongly conserved throughout eukaryotes, suggesting an important function for this subunit, possibly in the regulation of the ATPase or ATP synthase activities of the enzyme. We considered it is plausible that dimerization of the F_0-sectors, mediated by Su e, may serve to keep two F_1-domains in close proximity of each other. A close spatial arrangement of one F_1-sector with another may support binding of the IF_1/Inh1 inhibitor protein, under conditions when the ATP hydrolysis activity of the enzyme requires regulation. Measurement of the ATPase activity under conditions of low $\Delta\mu H^+$ and matrix acidification indicated that hydrolysis activity was, however, not induced in the Δ*su e* null mutant mitochondria. Thus, the inhibition of ATP hydrolysis activity by Inh1 of the monomeric ATP synthase in the Δ*su e* mitochondria was as effective as that of the dimeric complex in wild-type mitochondria. Furthermore, the efficient inhibition of the ATP hydrolysis activity was not achieved through a compensatory increase in the levels of Inh1 or Stf1 in the Δ*su e* null mutant mitochondria, relative to wild type. We conclude therefore that the formation of Su e-mediated F_1F_0-ATP synthase dimers is not required for inhibition of the ATPase activity by the natural inhibitor protein Inh1. Indeed, our analysis has indicated that the Inh1 protein is found in association with both the dimeric and monomeric forms of the ATP synthase.

Paumard and colleagues (27) reported recently that neighboring ATP synthase dimers interact together to form a larger network of oligomeric structures, which appear to modulate the morphology of the mitochondrial cristae. This ATP synthase dimer-dimer interaction is proposed to occur through interactions between one Su 4 of the asymmetrically located stator complex from one F_1F_0-ATP synthase complex with another Su 4 protein from a neighboring complex (28). Thus the Su e-Su e-mediated ATP synthase dimers would be interconnected with each other through these Su 4-Su 4 interactions. Consequently, one of the functions of the Su e-mediated ATP synthase dimers would appear to be to enable the formation of this oligomeric network. Whether such an oligomeric network functions to regulate the enzymatic activity of the ATP synthase or the mitochondrial energetic state, independently or dependently of its affect on the cristae morphology, awaits further investigation.

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