Su e of the Yeast F₁F₀-ATP Synthase Forms Homodimers*

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The yeast F₁F₀-ATP synthase forms a dimeric complex in the mitochondrial inner membrane. Dimerization of two F₁F₀, monomeric complexes involves the physical association of two membrane-embedded F₀ sectors and in a manner, which is dependent on the F₁ subunit, Su e. Sequence analysis of Su e protein family members indicated the presence of a conserved coiled-coil motif. As this motif is often the basis for protein homodimerization events, it was hypothesized that Su e forms homodimers in the inner membrane and that formation of Su e dimers between two neighboring F₁ complexes would facilitate dimerization of the F₁F₀-ATP synthase complex (Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., and Schägger, H. (1998) EMBO J. 17, 7170–7178). Using a histidine-tagged derivative of yeast Su e, Su e-His₁₂, combined with cross-linking and affinity purification approaches, we have directly demonstrated the ability of the yeast Su e protein to form homodimers. Functionality of the Su e-His₁₂ derivative was confirmed by its ability to assemble into the ATP synthase complex and to support its dimerization in the Δsu e null mutant yeast cells. The close association of two neighboring Su e proteins was also demonstrated using cross-linking with Cu²⁺, which binds and cross-links a unique Cys residue in neighboring Su e proteins. Finally, we propose a model for the molecular basis of the homodimerization of the Su e proteins.

Mitochondria, eukaryotic organelles, produce energy in the form of ATP (adenosine 5'-triphosphate) by a process termed oxidative phosphorylation. The F₁F₀, ATP synthase complex catalyzes the formation of this ATP from ADP (adenosine 5'-diphosphate) and in a manner that is coupled to the transport of protons across the inner membrane from the intermembrane space to the matrix (1–3). In general, F₁F₀-ATP synthase complexes may be resolved into two oligomeric parts, the catalytic F₁ sector, which performs the ATP synthesis and hydrolysis reactions, and the membrane-embedded F₀ sector, which mediates the proton transport steps. Both the F₀ and the F₁ sectors are multisubunit protein complexes. The F₁ sector is composed entirely of nuclear-encoded subunits, whereas the membrane-bound F₀ sector is assembled from both nuclear and mitochondrially encoded proteins (1–3). The mitochondrial F₁F₀-ATP synthase complexes do not exist as physically independent entities, but rather the complexes appear to be associated together as a larger oligomeric network of complexes in the inner mitochondrial membrane (4, 5). Consistent with an earlier hypothesis proposed by Allen (6), formation of this oligomeric F₁F₀-ATP synthase network has been shown to play a critical role in the modulation of the cristae morphology (5).

Formation of the ATP synthase oligomeric network appears to be dependent on the initial dimerization of two F₁F₀-ATP synthase complexes (5). Evidence for the formation of the mitochondrial F₁F₀-ATP synthase dimers, was first demonstrated in the yeast Saccharomyces cerevisiae mitochondria (4, 7, 8). The dimeric ATP synthase was initially identified following mild detergent solubilization of mitochondrial membranes and subsequent analysis by either size-exclusion chromatography or blue native gel electrophoresis (BN-PAGE) (4, 7). A comparison of the subunit composition of the dimeric and monomeric forms of the ATP synthase following the BN-PAGE led to the identification of a number of novel, dimer-specific subunits, termed e, g, and k (Su e, Su g, and Su k, respectively) (4). Deletion of the genes encoding Su e, Su g, or Su k, showed them to be all non-essential subunits of the F₁F₀-ATP synthase complex, as the resulting null mutants (Δsu e, Δsu g, and Δsu k, respectively) were viable on non-fermentable carbon sources indicating that their mitochondria were respiratory-competent (4). The Su e and Su g proteins, however, were shown to play an important role in the formation of the dimeric ATP synthase complex. As demonstrated by BN-PAGE analysis, formation of the dimeric ATP synthase was adversely affected in mitochondria isolated from the Δsu e or Δsu g null mutants (4). Formation of the Su e-mediated F₁F₀ dimers appears to be a prerequisite for the assembly of the F₁F₀ complexes into the larger oligomeric network (5). Failure to form this complex network in the Δsu e null mutant resulted in morphological changes of the mitochondrial inner membrane, with a notable absence of the characteristic inner membrane cristae tubular network (5).

The presence of F₁F₀-ATP synthase dimers has subsequently been characterized in a number of eukaryotic mitochondria, including bovine and human (9). Consistently, the Su e and Su g proteins are conserved throughout eukaryotic evolution, found both in fungal and mammalian mitochondria. Su e and Su g are integral inner membrane proteins, indicating them to be components of the membrane-embedded F₀ sector (4, 7). Consequently, dimerization of the yeast F₁F₀-ATP synthase had been proposed to involve the physical association of two membrane-embedded F₀ sectors, and in a manner that involves the Su e and Su g proteins (4). Previous work has supported a central role of Su e in this F₁F₀-ATP synthase dimerization

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event (4). Su e spans the inner membrane once via a single transmembrane segment, located at the extreme N-terminal region (approximately residues 1–25). The C-terminal region of Su e, the bulk of the protein (approximately 70 residues), thus protrudes into the intermembrane space (7). Sequence analysis of known Su e-family members has indicated the presence of a conserved putative coiled-coil motif in the Su e protein, which is located directly C-terminal to the transmembrane segment (7). As coiled-coil motifs can often be the basis for homodimerization events, we previously proposed that Su e protein forms homodimers in the inner membrane (4). Furthermore, formation of Su e-Su e homodimers, between two neighboring F_{1}O_{1} complexes was hypothesized to play an important role in facilitating dimerization of the F_{1}O_{1}-ATP synthase complex (4). In support of this model, it was previously shown that bovine Su e could be chemically cross-linked to a protein of a similar size to Su e, thus providing preliminary support for Su e-Su e dimers (10). Direct demonstration of homodimers of Sue, however, has been lacking to date.

In this present study we directly analyzed the ability of the yeast Su e protein to form homodimers in the mitochondrial inner membrane. We have expressed a histidine-tagged derivative of yeast Su e, Su e-His_{12} in either wild type or Δsu e null mutant yeast cells. We demonstrate the functionality of the tagged Su e derivative by its ability to assemble into the ATP synthase complex and support dimerization of the complex. Using a chemical cross-linking and affinity purification approach, we directly demonstrate the formation of Su e-Su e dimers in the mitochondrial membrane. The close association of two neighboring Su e proteins is also demonstrated using cross-linking with Cu^{2+}, which binds and cross-links a single Cys residue in neighboring Su e proteins. Finally, taking this information together, we propose a model for the molecular basis of the homodimerization of the F_{1}O_{1}-ATP synthase Su e protein.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Yeast strains used in this study were wild type (WT) W303–1A (MAT a, leu2, trp1, ura3, his3, ade2) and the su e null mutant, Δsu e (W303–1A, leu2, trp1, ura3, ade2, TIM11:HIS3) (4, 11). Mitochondria were isolated from the resulting yeast strains, which had been grown in YP (yeast extract, peptone)-Gal medium (2% galactose) supplemented with 0.5% lactate (12). Samples containing the 2μ Yep51-Su e-His_{12} plasmid (see below) were grown in the absence of added leucine on selective medium (SD(−Leu,−Trp,−Ade)) to which 0.1% galactose was added (0.1% galactose). The C-terminal region of Su e protein (7).

**Protein purification of the histidine-tagged Su e protein using Ni-NTA beads**—Mitochondria (600 μg of protein) were resuspended in 600 μl of digitonin-lysing buffer (1% (w/v) digitonin, 150 mM potassium acetate, 30 mM Hepes (pH 7.4), 10 mM imidazole, 1 mM PMSF) for 30 min on ice. Following lysis, 600 μl of lysis buffer without digitonin was added, and the samples were subjected to a clarifying spin by centrifugation for 30 min, at 226,000 × g (TLA45 rotor, Beckman TL-100 ultracentrifuge). The supernatant was then added to 90 μl of Ni-NTA-agarose bead suspension, which had been washed previously in the digitonin-lysing containing buffer. Binding was performed for 60 min at 4 °C under constant gentle rotation. The Ni-NTA beads were recovered by centrifugation, the supernatant was removed, and the non-bound proteins were precipitated with the addition of trichloroacetic acid. The beads were washed twice with the digitonin-lysing buffer, and the bound proteins were eluted following the addition of SDS-containing sample buffer containing 400 mM imidazole. Samples were shaken for 10 min at 4 °C and then were heated at 95 °C for 3 min. Following centrifugation the supernatant was removed and analyzed by SDS-PAGE and Western blotting. Immunodecoration of the resulting blot with Su e and F_{1} α-subunit-specific antisera was performed.

**Affinity Purification of Su e-His_{12} and Cross-linked Adducts on Ni-NTA-Agarose following Lysis under Denaturing Conditions**—Following cross-linking with DTNB (0.2 mM, see above), mitochondria (1.5 mg) were solubilized in 150 μl of SDS-lysis buffer (1% SDS, 0.1 M Tris-Cl (pH 7.4), 2 mM PMSF) for 10 min at 4 °C and then were heated to 95 °C for 3 min. The SDS-lysed mitochondria were diluted 20-fold with a Triton X-100-containing buffer (0.5% Triton-X-100, 30 mM Tris-Cl (pH 7.4), 10 mM imidazole, 1 mM PMSF). Following centrifugation for 15 min at 30,000 × g (4 °C, Sigma, Rotor 12154), the supernatants were added to 90 μl of Ni-NTA-agarose bead suspension. The binding and elution of the Su e-His_{12} and cross-linked adducts was then performed, essentially as described above.

**BN-PAGE Analysis of the Dimeric F_{1}F_{0}-ATP Synthase Synthase Forms Homodimers**—Chemical cross-linking of Su e protein with Cu^{2+}—Isolated mitochondria (100 μg of protein) were lysed in 40 μl of digitonin buffer (1% (w/v) digitonin, 50 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 10% glicerol 1 mM PMSF) for 30 min on ice and subjected to a clarifying spin (30 min, 226,000 × g, TLA45 rotor, Beckman TL-100 ultracentrifuge). The mitochondrial pellets were suspended with 4 μl of sample buffer (5% (w/v) Serva Blue G in 500 mM aminocaproic acid) prior to electrophoresis. Samples were then analyzed by BN-PAGE using 5–10% polyacrylamide gradient gels, essentially as described previously (4, 14). Following electrophoresis, Western blotting to nitrocellulose was performed, and protein complexes were detected by immunodecoration. The calibration standards used in the BN-PAGE are as follows: bovine thyroglobulin (670 kDa), horse spleen apoferritin (443 kDa), and bovine serum albumin monomer (66 kDa). The dimeric and monomeric forms of the Su e protein were detected by immunodecoration with F_{1} α-subunit-specific antisera.

**Cross-linking of Su e Protein with Cu^{2+}—**Isolated mitochondria (100 μg of protein) were resuspended in SH buffer (0.6 M sorbitol, 20 mM Hepes, pH 7.5) at a protein concentration of 0.5 mg/ml, and cupric sulfate (CuSO_{4}) cross-linking was performed essentially as previously described (15). CuSO_{4} was added at concentrations ranging from 0–10 μM, as indicated, and samples were incubated on ice for 30 min. Free sulphydryl residues were then blocked by the addition of the cysteine-modifying reagent N-ethylmaleimide (10 mM), and excess Cu^{2+} was quenched following the addition of EDTA (10 mM). Samples were further incubated on ice for 15 min, and mitochondria then were reisolated by centrifugation. Mitochondria were lysed with SDS-containing sample buffer containing N-ethylmaleimide and EDTA (both 10 mM) in the presence of the remaining β-mercaptoethanol, which had been described (13) and leucine positive transfectants were selected. Expression of Su e-His_{12} in the wild type (WT + Su e-His_{12}) and the su e null mutant background (Δsu e + Su e-His_{12}) were verified by Western blotting.

**Chemical Cross-linking of Su e Protein with DTNB—Isolated mitochondria (100 μg of protein) were resuspended in SH buffer (0.6 M sorbitol, 20 mM Hepes, pH 7.2) and incubated at 95 °C for 5 min. Samples were analyzed by SDS-PAGE using a 16% acrylamide, 0.6% bisacrylamide gel followed by Western blotting.

**Affinity Purification of Su e-His_{12} on Ni-NTA-Agarose following Lysis under Native Conditions**—A batch method was used for the affinity purification of the histidine-tagged Su e protein using Ni-NTA beads (Figure 1A). Mitochondria (600 μg of protein) were resuspended in 600 μl of digitonin-lysing buffer (1% (w/v) digitonin, 150 mM potassium acetate, 30 mM Hepes (pH 7.4), 10 mM imidazole, 1 mM PMSF) for 30 min on ice. Following lysis, 600 μl of lysis buffer without digitonin was added, and the samples were subjected to a clarifying spin by centrifugation for 30 min, at 226,000 × g (TLA45 rotor, Beckman TL-100 ultracentrifuge). The supernatant was then added to 90 μl of Ni-NTA-agarose bead suspension, which had been washed previously in the digitonin-lysing containing buffer. Binding was performed for 60 min at 4 °C under constant gentle rotation. The Ni-NTA beads were recovered by centrifugation, the supernatant was removed, and the non-bound proteins were precipitated with the addition of trichloroacetic acid. The beads were washed twice with the digitonin-lysing buffer, and the bound proteins were eluted following the addition of SDS-containing sample buffer containing 400 mM imidazole. Samples were shaken for 10 min at 4 °C and then were heated at 95 °C for 3 min. Following centrifugation the supernatant was removed and analyzed by SDS-PAGE and Western blotting. Immunodecoration of the resulting blot with Su e and F_{1} α-subunit-specific antisera was performed.

**RESULTS**

Su e Plays an Essential Role in the Dimerization of the F_{1}F_{0}-ATP Synthase Synthase—The assembly state of the F_{1}F_{0}−ATP synthase was analyzed in mitochondria isolated from both the su e and su g null mutant yeast strains, together with those isolated from the isogenic wild type strain (Fig. 1A). Following solubilization with digitonin, mitochondrial protein complexes were resolved by BN-PAGE followed by Western blotting. Im-
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Fig. 1. The presence of Su e is essential for the formation of the F₁F₄-ATP synthase dimer. A, mitochondria isolated from wild type yeast (WT) or from yeast mutants deficient in either Su e (Δsu e) or Su g (Δsu g) were solubilized in digitonin as described under "Experimental Procedures." The dimeric state of the F₁F₄-ATP synthase was analyzed by BN-PAGE followed by Western blotting and decoration with an antiserum specific for the α-subunit of the F₁ sector. The positions of the dimeric (Vdim.) and the monomeric (Vmon.) ATP synthase and the free F₁ sector are indicated. B, mitochondria isolated from the Δsu e, Δsu g, and the Δsu k null mutant strains and corresponding wild type (WT) strain were subjected to SDS-PAGE and analyzed by Western blotting for the presence of Su e and Su k proteins.

null mutant demonstrated that the Su e protein was detectable in the Δsu g null mutant mitochondria, although strongly reduced relative to the wild type control mitochondria (Fig. 1B). Thus reduced ability of the ATP synthase to form dimers in the Δsu g mitochondria is most likely directly related to the reduced levels of Su e in these mitochondria (Fig. 1B). In addition the levels of Su k were strongly reduced, yet still detectable, in both the Δsu e and Δsu g mitochondria (Fig. 1B). Thus, although not essential for Su k, the presence of Su g and Su e appear to enhance the steady state levels of Su k. Thus taken together, we conclude that the Su e protein plays the central role in forming the F₁F₄-ATP synthase dimer and that Su g protein plays an accessory role, which may involve a stabilization effect on the Su e protein.

Expression and Functionality of the Su e-His₁₂ Derivative—To facilitate the further characterization of Su e, we constructed a derivative of Su e, which contained an additional 12 histidine residues at the C terminus of the protein. Expression of the resulting histidine-tagged Su e, Su e-His₁₂, was achieved by cloning the extended Su e open reading frame encoding Su e-His₁₂ into a 2μ vector, Yep51, which contained a galactose-inducible GAL10 promoter. The Su e-His₁₂ protein was expressed both in the wild type (WT + Su e-His₁₂) and su e null mutant, Δsu e, Δsu e + Su e-His₁₂, genetic backgrounds. Western blot analysis of mitochondria isolated from the WT + Su e-His₁₂ and Δsu e + Su e-His₁₂ strains confirmed the expression of the Su e-His₁₂ derivative (Fig. 2A).

Following the successful expression of Su e-His₁₂, our initial experiments were aimed at demonstrating that the addition of the histidine tag to Su e did not functionally compromise the Su e protein. To do so, mitochondria were isolated from the Δsu e null mutant, Δsu e + Su e-His₁₂ strain, and the isogenic wild type background, and the steady state levels of the ATP synthase subunits Su g and Su k were analyzed. As previously described, the levels of Su g and Su k are significantly decreased in the absence of Su e, i.e. in the Δsu e mitochondria (4). The functionality of the expressed Su e-His₁₂ was indicated from its ability to restore the levels of Su g and Su k in the Δsu e mitochondria (Fig. 2B).

Functionality of the histidine-tagged Su e derivative was further tested by its ability to support the formation of the dimeric ATP synthase in the su e null mutant mitochondria (Fig. 2C). BN-PAGE analysis indicated the presence of the dimeric form of the F₁F₄-ATP synthase in the Δsu e + Su e-His₁₂ mitochondria. Furthermore, expression of the Su e-His₁₂ protein appeared to support the stabilization of the F₁F₄-ATP synthase by the noticeable lack of the free F₁ sector in the Δsu e + Su e-His₁₂ mitochondria, in contrast to the su e null mutant mitochondria and lacking the Su e-His₁₂ protein (Fig. 2C).

Assembly of Su e-His₁₂ into the F₁F₄-ATP Synthase Complex—The functionality of the Su e-His₁₂ derivative was further directly demonstrated by testing its ability to assemble together with other F₁F₄-ATP synthase complex subunits. Su e-His₁₂ was expressed in wild type yeast cells, and the mitochondria were isolated from these cells (WT + Su e-His₁₂). Mitochondrial membrane proteins were solubilized with digitonin, a mild detergent known to maintain the assembled dimeric state of the F₁F₄-ATP synthase complex. Following solubilization in this manner, Su e-His₁₂ was affinity-purified via its histidine tag on a nickel-containing agarose matrix (Ni-NTA beads). Western blot analysis of the material bound to the Ni-NTA beads indicated that in addition to the Su e-His₁₂ protein both wild type Su e and the α subunit of the F₁ sector (F₁ α) had been co-purified. The co-purification of F₁ α and Su e on the Ni-NTA beads was dependent on the presence of the Su e-His₁₂ protein as they were not observed bound to the Ni-NTA beads, following
histidine-tagged derivative indicates that the F1Fo complex

tantly, the co-purification of the wild type Su e protein with the

presence of Su e and the Su e-His 12 derivative using a Su e-specific

subjected to SDS-PAGE and analyzed by Western blotting for the

additional Su e subunit. Indeed, preliminary stoichiometric

analysis of the subunit composition of the dimeric F1F0-ATP synthase had suggested two Su e protein subunits/dimeric synthase (4).

Su e Forms a Homodimer—As previously mentioned the Su e proteins contain a conserved predicted coiled-coil motif, often the basis for homodimerization events. This, together with the observation that the Su e-His12-purified F1F0-ATP synthase complex contains more than one subunit of the Su e protein, led us to test the ability of Su e to form homodimers. We initially tested the ability of Su e (11 kDa) to be cross-linked to another protein of the same size. To do so, mitochondria were subjected to chemical cross-linking using DTNB, Ellman's reagent, a sulfhydryl-specific, reductant cleavable cross-linking reagent. Yeast Su e contains a single Cys residue (Cys-28), which according to hydropathy plots, is located proximal to the C-terminal end of the single transmembrane region of Su e, i.e. at the intermembrane space-side of the inner membrane. Following cross-linking, samples were analyzed by SDS-PAGE in the absence of a reducing agent and then by Western blotting. Using the Su e-specific antibody, Su e and resulting cross-linked adducts were identified (Fig. 4A). When cross-linking was performed in wild type mitochondria, a Su e cross-linked adduct of approximately 22 kDa, the predicted size for a Su e-Su e homodimer was observed. Cross-linking in Δsu e + Su e-His12 mitochondria, resulted in the production of a 26-kDa adduct, a size in agreement for a Su e-Su e-His12 homodimer. Finally, cross-linking of Su e in the wild type mitochondria harboring also the Su e-His12 protein (WT + Su e-His12) resulted in a mixed population of Su e-specific adducts of predicted sizes, we postulate these three adducts to correspond to Su e-Su e, Su e-Su e-His12, and Su e-His12-Su e-His12 homodimers, respectively.

To confirm directly that Su e does indeed form homodimers, wild type mitochondria harboring the Su e-His12 protein were subjected to cross-linking with DTNB. Following cross-linking, the mitochondrial membranes were solubilized with the denaturing detergent SDS to disrupt any non-cross-linked complexes, and the Su e-His12 protein and cross-linked partners were affinity-purified on Ni-NTA-agarose beads. Affinity-purified Su e and the cross-linked proteins were then incubated with the reducing β-mercaptoethanol to cleave the chemical cross-linker and were subsequently analyzed by SDS-PAGE

their solubilization from wild type mitochondria, which did not harbor the Su e-His12 protein (Fig. 3).

In conclusion, these data confirm the ability of the Su e-His12 to assemble into the F1F0-ATP synthase complex. Most importantly, the co-purification of the wild type Su e protein with the histidine-tagged derivative indicates that the F1F0 complex containing the Su e-His12 protein must contain at least one additional Su e subunit. Indeed, preliminary stoichiometric
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**FIG. 4.** Chemical cross-linking of Su e-Su e homodimers. A, mitochondria (100 µg of protein) isolated from the wild type (WT) and the Δsu e null mutant strains and the corresponding strains expressing the Su e-His12 derivative, WT + Su e-His12 and Δsu e + Su e-His12, respectively, were treated with the chemical cross-linker DTNB (0.4 mM) or were mock-treated and received Me2SO (-), as described under "Experimental Procedures." Following cross-linking, mitochondria were resolubilized by centrifugation and subjected to non-reducing SDS-PAGE and Western blotting. Su e, Su e-His12, and their cross-linked adducts were identified following decoration of the resulting blot with Su e-specific antisera. Adducts with electrophoretic mobilities corresponding to Su e-Su e (+), Su e-Su e-His12 (−, Δsu e), and Su e-His12-Su e-His12 (Δsu e, Cys residue of the interacting Su e molecule. A 17-kDa protein that cross-reacts with the Su e antisera (present also in the Δsu e null mutant) is indicated (*). B, following cross-linking with DTNB (0.2 mM, see above), wild type (WT) mitochondria or wild type harboring Su e-His12 (WT + Su e-His12) (1.5 mg of protein) were solubilized in SDS-lysis buffer, as described under "Experimental Procedures." Following dilution with a Triton X-100-containing buffer and a clarifying spin, the solubilized proteins were incubated with Ni-NTA-agarose beads. The binding and elution of the Su e-His12 and cross-linked adducts was performed essentially as described above in Fig. 3.

**FIG. 5.** Cu2+ cross-linking of Su e-Su e homodimers. Mitochondria (100 µg of protein) were incubated for 30 min on ice with increasing concentrations of CuSO4 as indicated. Following the addition of N-ethylmaleimide, EDTA, and cysteine, (each 10 mM), mitochondria were resolubilized by centrifugation, washed, and lysed in non-reducing SDS-containing sample buffer that contained N-ethylmaleimide and EDTA (each 10 mM). Samples were then analyzed by SDS-PAGE, Western blotting, followed by immunodecoration with Su e-specific antisera. A Su e-cross-linked adduct with an electrophoretic mobility corresponding to a Su e-Su e homodimer (Δsu e) is indicated.

Presumably the endogenous divalent cation level (no EDTA was present) may have supported the formation of this adduct. The mobility of the Su e-Cu2+ cross-linked adduct was similar to that of the Su e-Su e homodimer, which had been cross-linked with DTNB. Taken together with the DTNB cross-linking data, we conclude therefore that the Su e forms homodimers, which are arranged in such a manner that the single Cys residue present in Su e is in close proximity to the Cys residue of the interacting Su e molecule.

**DISCUSSION**

The F1F0-ATP synthase forms dimeric complexes in the mitochondrial membrane (4, 7). Evidence that these F1F0-ATP synthase dimers further assemble together to form a larger network of F1F0 complexes in the mitochondrial inner membrane was recently presented (5). Our current data indicates that the initial ATP synthase dimerization event is supported through a physical interaction of neighboring F0 sectors. We have recently demonstrated that dimerization of the F1F0-ATP synthase in intact mitochondria does not involve the natural inhibitor protein, Inh1, which was recently shown to bind to and promote dimerization of purified F1 sectors in solution (18–20). Rather, our data indicate that subunits e and g, Su e and Su g, are required for efficient ATP synthase dimer formation. In the absence of Su e, no dimer was observed and the F1F0-ATP synthase was present exclusively in its monomeric form. In contrast, in the Δsu g mutant mitochondria, although strongly reduced in levels, a small percentage of F1F0 complexes were present as dimers. The levels of Su e are strongly reduced (yet still detectable) in the Δsu g mitochondria, thus indicating that Su g is involved in the stability of the Su e protein. We propose that Su e plays a central role in the formation of the dimeric F1F0-ATP synthase and that Su g plays a supporting role and one that involves stabilization of the Su e protein.

Su e family members contain a conserved coiled-coil motif in their hydrophilic region, just C-terminal to their transmembrane-spanning segments (7). As coiled-coil motifs are often the basis for protein-protein interactions, in particular homodimerization events, we proposed that the Su e mediated dimerization of the F1F0-ATP synthase complex involved the formation of Su e-Su e homodimers (4). Preliminary evidence for the presence of the Su e-Su e homodimers was reported earlier as the bovine Su e protein could be cross-linked to another protein of a molecular mass similar to that of Su e itself (10). Here, we have extended that initial observation and, using a chemical cross-linking approach combined with affinity purification, we...
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have directly demonstrated the formation of Su e-Su e homodimers in the yeast mitochondrial membrane. We have expressed a histidine-tagged Su e derivative, Su e-His12, in both wild type and Δsu e mitochondria. The functionality of this Su e-His12 derivative was shown 3-fold: (i) by its ability to restore steady-state levels of Su g and Su k when expressed in the Δsu e yeast strain; (ii) by its ability to assemble and co-purify with the F1F0-ATP synthase complex; and finally, (iii) by its ability to support the dimerization of the ATP synthase in the Δsu e mitochondria. When expressed in wild type cells, Su e-His12 directly assembles and homodimerizes with the authentic Su e protein. Dimerization of Su e-His12 with Su e was directly demonstrated by chemical cross-linking followed by their co-purification on Ni-NTA agarose.

What is the molecular basis for the Su e-Su e homodimerization? The dimerization of the F1F0-ATP synthase is a conserved feature between the mitochondrial F1F0-ATP synthase complexes, and hence it is most likely to be supported by a conserved feature of the Su e protein family. For this reason, we consider it unlikely the Su e-Su e interaction is mediated by the extreme C-terminal region of Su e (the last approximately 30 amino acid residues). This C-terminal region (residues 70–96) of the protein is unique to the yeast Su e homolog (7). Furthermore, the cross-linking of Su e proteins with Cu2+ ions or with DTNB indicates that the unique Cys residue in Su e is in close proximity to the Cys of the associated Su e protein. As the Cys residue is located immediately after the transmembrane-spanning segment and at the interface with the coiled-coil region, it implies that these regions of the Su e polypeptide must be physically close in the Su e dimer. As mentioned previously, the predicted coiled-coil motif is a conserved feature between Su e protein family members. It is therefore highly possible that this region plays an important role in Su e function. Taking this together with the known involvement of coiled-coil regions in protein homodimerization events, it is possible that the Su e homodimerization involves coiled-coil interactions between neighboring Su e proteins. Alternatively, it is possible that the Su e homodimerization process involves helix-helix interactions between the transmembrane helices of interacting Su e subunits. Indeed a conserved GXXXG (G for glycine and X for any amino acid residue) motif has been described to be a frequently occurring sequence of residues that favor helix-helix interactions of transmembrane segments of dimerizing membrane proteins (21–23). Interestingly, a GXXXG motif is found in the transmembrane segment of the yeast Su e protein. The possible importance of this GXXXG motif in Su e-Su e homodimerization events is further suggested by the fact that the GXXXG motif is conserved between the transmembrane regions of the other Su e family members also. Thus taken together, it is plausible that the coiled-coil motif and/or the GXXXG motif in the transmembrane-spanning segment, rather than the extreme C-terminal region of Su e, play a direct role in the homodimerization of Su e. Experiments designed to test these possibilities are currently ongoing in our laboratory.

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