The Yeast F₁F₀-ATP Synthase

ANALYSIS OF THE MOLECULAR ORGANIZATION OF SUBUNIT g AND THE IMPORTANCE OF A CONSERVED GXGXG MOTIF

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The F₁F₀-ATP synthase enzyme is located in the inner mitochondrial membrane, where it forms dimeric complexes. Dimerization of the ATP synthase involves the physical association of the neighboring membrane-embedded F₀-sectors. In yeast, the F₀-sector subunits g and e (Su g and Su e, respectively) play a key role in supporting the formation of ATP synthase dimers. In this study we have focused on Su g to gain a better understanding of the function and the molecular organization of this subunit within the ATP synthase complex. Su g proteins contain a GXGXG motif (G is glycine, and X is any amino acid) in their single transmembrane segment. GXGXG can be a dimerization motif that supports helix-helix interactions between neighboring transmembrane segments. We demonstrate here that the GXGXG motif is important for the function and in particular for the stability of Su g within the ATP synthase. Using site-directed mutagenesis and cross-linking approaches, we demonstrate that Su g and Su e interact, and our findings emphasize the importance of the membrane anchor regions of these proteins for their interaction. Su e also contains a conserved GXGXG motif in its membrane anchor. However, data presented here would suggest that an intact GXGXG motif in Su g is not essential for the Su g–Su e interaction. We suggest that the GXGXG motif may not be the sole basis for a Su g–Su e interaction, and possibly these dimerization motifs may enable both Su g and Su e to interact with another mitochondrial protein.

The F₁F₀-ATP synthase enzyme plays a pivotal role in the aerobic production of ATP (adenosine triphosphate) in eukaryotic cells. This enzyme is located in the inner membrane of mitochondria and is composed of a large number of different subunits (1–4). Functionally, the enzyme can be divided into two parts or subcomplexes, the inner membrane embedded F₀-sector and the associated hydrophobic F₁-sector, which protrudes into the mitochondrial matrix. The F₁-sector is responsible for the passage of protons from the intermembrane space into the matrix, a step coupled to the F₁-catalyzed synthesis of ATP from ADP (adenosine diphosphate) and phosphate (4).

In mitochondria the F₁-sector is composed of a number of different polypeptides, which are both nuclear- and mitochondria-encoded. In yeast, for example, at least 10 different subunits make up this subcomplex, three of which (subunits 6, 8, and 9) are encoded by the mitochondrial DNA (mtDNA)1 (2, 3). The F₀-sector of the mitochondrial enzyme is, thus, more complex than that of its bacterial counterpart, where only three subunits (subunits a, b, and c) have been described. In addition to mediating the passage of H⁺ ions across the inner membrane, the F₀-sector also has been described to physically associate with neighboring F₀-sectors, which results in the dimerization of ATP synthase complexes in the mitochondrial inner membrane (5–15). Three F₀-sector subunits, subunit 4 (Su 4, equivalent to bacterial subunit b) and the two recently identified subunits, subunits e and g (Su e and Su g, respectively) have been shown to play a critical role in the process of F₀-sector dimerization (5–8, 15–18). A number of independent lines of evidence for the ATP synthase dimerization have been presented in the literature over the recent years. First, molecular sizing analysis, either gel filtration or native gel electrophoresis of the ATP synthase complexes solubilized from mitochondrial membranes with a mild detergent, demonstrated that the ATP synthase can be isolated as a complex whose mass (~1000 kDa) was consistent with that of a dimeric complex (5, 6, 9, 14). Subsequent chemical cross-linking approaches adopted by Velours and co-workers (7, 17), demonstrated the close physical proximity of two Su 4 proteins in the mitochondrial inner membrane. Because the stoichiometry of Su 4 is known to be 1 Su 4 per F₀-sector, the observed Su 4–Su 4 cross-linking provides evidence for F₀-F₀ interaction in the mitochondrial membrane (7, 17). More recently, in vivo studies using fluorescent resonance energy transfer technology has also supported a close physical association of one F₁F₀-ATP synthase complex with another (12, 13).

Gene knock-out studies in yeast combined with biochemical analysis of the ATP synthase complex have indicated that the conserved Su e and Su g proteins are not essential for the enzymatic activity of the complex (6, 9, 17). The ATP synthase capacity of the complex has been reported to be partially compromised in the absence of Su e and Su g, however (9, 14, 18), and consequently, the null mutants exhibit a weak growth phenotype on non-fermentable carbon sources (6, 9, 18, 19). Further analysis of the mitochondria from the null mutant strains, Δsu e and Δsu g, respectively, however, revealed that these mutants exhibit two interesting phenotypes. First, in the absence of Su e and/or Su g, the ATP synthase complex failed to assemble into detergent stable dimers, as indicated by native gel electrophoresis (6, 9, 14, 15). However, Su 4–Su 4 cross-linking has been reported in Δsu e and Δsu g mitochondria, indicating that a close physical proximity of ATP synthase complexes can still exist in the absence of Su e and Su g.
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proteins (9). These Su 4-Su 4-mediated interactions appear not to be stable in the absence of Su e and Su g, however, as only monomeric ATP synthase complexes are observed after detergent solubilization (6, 9, 14, 15). Second, the importance of the Su g and Su e proteins have been further highlighted by the recent demonstration that the presence of these proteins are required to develop normal mitochondrial morphology, in particular development of the characteristic cristae structure of the inner membrane (9, 14, 16). Cristae are finger-like invaginations of the inner mitochondrial membrane, protruding into the mitochondrial matrix and are distinct from the inner boundary membrane, which is in close proximity to the outer mitochondrial membrane. In the absence of Su g or Su e, the mitochondrial inner membrane fails to partition into cristae invaginations but, rather, accumulates as onion-type membranes (9, 14, 16).

To further understand how Su e and Su g may affect the ATP synthase and its organization in the inner mitochondrial membrane, it is important to gain a better understanding of the molecular environment of these subunits within the ATP synthase and to identify the proteins they interact with. This present study focuses on the Su g protein. Su g is a relatively small protein (115 amino acid residues) and spans the inner mitochondrial membrane in an N to C orientation (6, 19). Su g has a single transmembrane segment (residues 88–106) that is located close to the C-terminal end of the protein (Fig. 1A). The bulk of the protein, the N-terminal hydrophilic domain (residues 1–87), is located in the mitochondrial matrix (Fig. 1A) (6). Alignment of Su g amino acid sequences indicates a high level of conservation in the C-terminal region of Su g, in particular the transmembrane segment (Fig. 1B). A tyrosine residue (Tyr-112) located immediately after the transmembrane region and two glycine residues (Gly-101 and Gly-105) within the transmembrane segment are conserved in all known Su g proteins (20). These conserved glycine residues form a motif GXXXG (G is Gly, and X represents any amino acid), which has been shown to be involved in the homo- or heterodimerization of other membrane proteins, e.g. glycophorin A (21–25). GXXXG motifs can form the framework for transmembrane helix-helix interactions, where the Gly residues and the spacing of three residues between them, have been shown to be essential aspects of the motif as they ensure a close helix-helix packing interface. The highly conserved nature of the GXXXG motif in Su g is indicative of its potential importance for the function of Su g, and it is possible that this motif may be involved in transmembrane helix-helix interactions between neighboring Su g proteins (homodimerization) or with another F1-sector subunit (heterodimerization).

Using site-directed mutagenesis combined with a cross-linking approach, we have addressed here the molecular environment of Su g within the F1Fo-ATPase complex. We provide evidence for a close proximity between Su g and the Su e protein and also for a putative Su g homodimerization. Furthermore, we show that alterations in the Su g protein can cause significant changes in the molecular environment of Su e. We also demonstrate the importance of the conserved GXXXG motif for the stability of Su g and its interaction with the ATP synthase.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Yeast strains used in this study were wild-type (WT) W303-1A (Mat a, leu2, trp1, ural3, his3, ade2) and the su g null mutant, Δsu g (W303-1A, leu2, trp1, ural3, ade2, ATP20::HIS3) (6). Mitochondria were isolated from the resulting yeast strains, which had been grown in YP (yeast extract, peptone) supplemented with 0.5% lactate and galactose (2%, YP-galactose medium) or glycerol (3%, YP medium), as indicated (26).

Cloning and Expression of Mutated and HA-Tagged Su g Derivatives—The entire open reading frame encoding Su g (ATP20 gene) was amplified by the PCR as a SpeI-PstI fragment. When indicated, the reverse primer used contained the sequence information to encode the hemagglutinin (HA) epitope before the translational stop codon in adenylate kinase (AK)-specific primer for cloning. ADH1 promoter, and the LEU2 auxotrophic marker gene (15, 27). In addition, Gly-101, Gly-105, Val-110, and Tyr-112 were individually mutated as indicated by incorporating the mutated sequence into the corresponding Su g reverse primer. The recombinant PCR products were cloned into a yeast integration vector Yip351, which contained a galactose-inducible GAL10 promoter, and the LEU2 auxotrophic marker gene (15, 27). The resulting plasmids were linearized at a unique ClaI restriction site located in the 5′ region of the LEU2 gene locus and transformed into the Δsu g null mutant using the protocol described by Knop et al. (28), and leucine-positive transformants were selected. Mitochondria were isolated from each of the transformants, and the expression and mitochondrial localization of the Su g derivatives were verified by Western blotting using an antibody specific for the HA epitope (Covance Research Products) or Su g antiserum (6).

Growth Curve Analysis—All strains were initially grown overnight at 30 °C in YPG medium supplemented with 0.2% galactose and histidine (0.06 mg/ml) and leucine (0.26 mg/ml). The cells were inoculated in the same medium at an A580 nm of 0.2, and growth of the cells was monitored by measuring the A580 nm at 2-h intervals over a period of 10 h.

rho0/rho + Cell Conversion Detection Assay—All yeast strains were maintained on YPG plates and were used to inoculate YP-lactate (0.5% lactate) supplemented with 2% galactose and allowed to grow overnight at 30 °C. The A580 nm of the overnight cultures was measured, and an equivalent number of cells from each culture was plated onto YPD (2% glucose) or YPG (3% glycerol, supplemented with 0.1% galactose) plates. After incubation at 30 °C, the colonies were counted, and the number of rho0/rho + cells (i.e. petite cells) was calculated and expressed as a% of total colonies on the YPD plate.

Chemical Cross-linking of Su g Protein with 5,5′-Dithiobis(2-nitrobenzoic Acid) (DTNB)—Isolated mitochondria (100 μg of protein) were resuspended in SH buffer (0.6 mM sorbitol, 20 mM Hepes, pH 7.2) at a protein concentration of 0.5 mg/ml. Cross-linking was performed on ice for 30 min in the presence of the sulfhydryl-specific homobifunctional reagent DTNB (0.2 mM). After quenching with 10 mM N-ethylmaleimide and 10 mM EDTA, pH 8.0, mitochondria were re-isolated by centrifugation, lysed in the presence of SDS-containing sample buffer (without β-mercaptoethanol), and heated at 95 °C for 5 min. Samples were analyzed by SDS-PAGE using a 16% acrylamide, 0.6% bisacrylamide gel followed by Western blotting (10).

Miscellaneous—Protein determinations and SDS-PAGE were performed according to published methods (29, 30). The Western blot analysis and antibody decoration were performed using available Su e, Su g, and Su k antisera raised against peptides corresponding to the C-terminal region of each of the proteins, respectively (6). The F1-antibody was raised in chicken after injection of purified yeast F1-sector and was obtained from Dr. David Mueller (The Chicago Medical School). Clear native-PAGE analysis of F1Fo-ATP synthase complexes after solubilization with digitonin was performed essentially as described previously (15).

RESULTS

Expression of the Mutated Su g Proteins—We adopted a cysteine mutagenesis and cross-linking approach to identifying proteins that may interact with Su g and also address the functional significance of the GXXXG motif in Su g. Su g contains one Cys residue at position 75, which is located in the matrix-exposed, hydrophilic region of Su g (Fig. 1A). We introduced an additional Cys residue into the C terminus of Su g, close to the transmembrane region and the conserved GXXXG motif. Two Su g mutants, Su gY112C and Su gV110C, were created whereby the Tyr and Val residues at positions 112 and 110 (Fig. 1, A and B) were individually mutated to Cys residues. We specifically chose to introduce the Cys residue into the C-terminal region of Su g as we wanted to analyze if Su g is in close proximity to Su e. Su e contains a unique Cys residue (Cys-27) immediately C-terminal to its membrane anchor segment, i.e. located in the intermembrane space, a position similar to that of the introduced Cys residues (V110C and Y112C) of Su g. The two resulting Su g derivatives, Su gY112C and Su gV110C, were cloned either in the absence or presence of an
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Yeast strains were grown at 30 °C on YPG medium supplemented with 0.2% galactose, histidine, and leucine. *rho*₀/rho⁺ production was measured as described under "Experimental Procedures." Additional C-terminal hemagglutinin tag (HA) tag (Su *g*Y112CHA and Su *g*V110CHA, respectively) and were expressed in a yeast *su g* null mutant. As a control we also expressed the wild-type Su *g* protein, at least under the conditions tested here.

### Phenotypic Analysis of Su *g* Derivative Strains

| Strain    | Doubling time | rho₀/rho⁺ cells |
|-----------|---------------|-----------------|
| Wild type | 131           | 1.1             |
| Δsu g     | 160           | 45.5            |
| Δsu g + Su HA | 123         | 1.0             |
| Δsu g + Su gY112C | 124     | 1.7             |
| Δsu g + Su gY112CHA | 120 | 4.9             |
| Δsu g + Su gV110CCHA | 121 | 2.5             |

Levels of both Su *g*Y112CHA and Su *g*V110CHA were similar to those of the wild-type HA-tagged construct. We, therefore, conclude that the alteration of residues Val-110 or Tyr-112 to cysteine did not hinder the mitochondrial targeting or stability of the Su *g* protein.

The steady state levels of other *F*₁-sector subunits, such as Su *e*, Su *k*, and Su *p* 4 proteins did not appear to be affected by the expression of the HA-tagged or mutated derivatives of the Su *g* protein (Fig. 1C). The levels of these proteins each appeared to be similar to those of wild-type mitochondria, thus demonstrating the assembly of the *F*₁-sector subunits was not hindered through the expression of the mutated Su *g* derivatives. In addition, growth curve analysis indicated that the strains harboring the Cys-modified Su *g* derivatives grew in a similar manner as the control wild-type strain on a non-fermentable carbon source, e.g. glycerol (Table I). As previously described the su *g* null strain, Δsu *g*, displayed a slightly slower growth phenotype under these conditions (6, 19).

Yeast cells lacking the Su *g* protein display an increased potential to undergo loss of their mtDNA (14, 18) (Table I). Expression of the wild-type Su *g* bearing the HA tag or the Su *g*V110CCHA and Su *g*Y112CHA derivatives in the su *g* null mutant background prevented the high frequency of spontaneous rho₀/rho⁺ formation, which was observed in the Δsu *g* strain in the absence of an expressed Su *g* protein (Table I).

Taken together these results suggest that introduction of a Cys residue at position 110 or 112 does not appear to adversely affect the stability of Su *g* protein. Thus, despite being conserved among the Su *g* protein family, mutation of residue Tyr-112 to a Cys does not appear to adversely affect the function of Su *g* protein, at least under the conditions tested here.

*Su g* and *Su e* Proteins Are Found in Close Proximity to Each Other—Cross-linking studies using the sulphydryl-specific reagent DTNB (Ellman's reagent) were performed with isolated mitochondria harboring the wild-type or the Cys-modified Su *g* derivatives. DTNB was chosen as a cross-linking agent due to the fact that it is a "zero-length" spacer arm cross-linking agent; thus, the ability to be cross-linked by DTNB is indicative of the close proximity of two neighboring sulphydryl residues. Wild-type control, Su *g*Y112C, or Su *g*Y112CHA mitochondria were incubated with DTNB or were mock-treated, and Su *g* and its cross-linked adducts were subsequently resolved on SDS-PAGE, Western-blotted, and subjected to immune-decoration with antibodies specific for Su *g* or the HA epitope (Fig. 2A). In the presence of DTNB, Su *g*Y112C formed a cross-linked adduct of ~23 kDa. This adduct corresponds to a product of Su *g* (~12 kDa) cross-linked to an ~11-kDa protein. The Su *g*Y112CHA derivative also formed a similar adduct in the presence of DTNB, which was ~24 kDa in mass, the difference in size between the Su *g*Y112C and Su *g*Y112CHA adducts accounted for by the presence of the HA tag (~1 kDa). In contrast the wild-type Su *g*, which lacks the additional Cys in the C-terminal region, did not form a similar cross-linked adduct.

**Table I**

| Strain | Doubling time | % of total |
|--------|---------------|------------|
| Wild type | 131           | 1.1         |
| Δsu g     | 160           | 45.5        |
| Δsu g + Su HA | 123         | 1.0         |
| Δsu g + Su gY112C | 124     | 1.7         |
| Δsu g + Su gY112CHA | 120 | 4.9         |
| Δsu g + Su gV110CCHA | 121 | 2.5         |

**Fig. 1.** Su *g* protein and expression of cysteine-modified derivatives. A, depiction of the Su *g* protein (115 amino acid residues in length). An open rectangle indicates the C-terminal hydrophilic transmembrane segment. QXXXG indicates the conserved motif located in the membrane anchor region (residues 88–106) of Su *g* (see "Experimental Procedures" for details). Cys-75, Val-110, and Tyr-112 indicate the membrane anchor region (residues 88–106) of Su *g* (see "Experimental Procedures" for details). Cys-75, Val-110, and Tyr-112 indicate the membrane anchor region (residues 88–106) of Su *g* (see "Experimental Procedures" for details). Cys-75, Val-110, and Tyr-112 indicate the membrane anchor region (residues 88–106) of Su *g* (see "Experimental Procedures" for details). Cys-75, Val-110, and Tyr-112 indicate the membrane anchor region (residues 88–106) of Su *g* (see "Experimental Procedures" for details). Cys-75, Val-110, and Tyr-112 indicate the membrane anchor region (residues 88–106) of Su *g* (see "Experimental Procedures" for details). Cys-75, Val-110, and Tyr-112 indicate the membrane anchor region (residues 88–106) of Su *g* (see "Experimental Procedures" for details). Cys-75, Val-110, and Tyr-112 indicate the membrane anchor region (residues 88–106) of Su *g* (see "Experimental Procedures" for details).
when incubated with DTNB (Fig. 2A). We, therefore, conclude that the Cys at position 112, located in the intermembrane space, is responsible for the cross-linking to the ~11-kDa protein. In addition, a cross-linked adduct of ~26 kDa, albeit with low efficiency, was observed in the Su gY112CHA derivates treated with the chemical cross-linker DTNB (+) or mock-treated and received Me$_2$SO (−), as described under "Experimental Procedures." After cross-linking and quenching of free DTNB, mitochondria were re-isolated by centrifugation, divided in half, and subjected to non-reducing SDS-PAGE and Western blotting. One of the resulting blots (A) was used to identify Su g and the Su gHA derivatives and their respective cross-linked adducts by decoration with Su g antisera or with HA-specific antisera, as indicated. Adducts with electrophoretic mobilities corresponding to Su e-Su g (e-g), Su e-Su gHA (e-gHA), and Su gHA-Su gHA (gHA-gHA) are indicated. Su e and cross-linked adducts were identified after decoration of the second blot with Su e-specific antisera (B, upper panel). Adducts with electrophoretic mobilities corresponding to Su e-Su e (e-e), Su e-Su g (e-g), Su e-Su gHA (e-gHA), and Su e-7 kDa (e-7kDa) are indicated. A protein that cross-reacts with the Su e antisemur (present also in the Su e null mutant (results not shown)) is indicated (*). B, lower panel, mitochondria (100 μg of protein) isolated from the wild-type (WT) and the Su gHA derivative. The observed difference in the cross-linking efficiency between Su gY112CHA and Su gY112CHA may be accounted for by a positioning effect of the residues. In the folded structure of Su g, residue Val-110 may be positioned away from the Cys-27 residue of Su e, whereas the residue 112 may be physically closer to it.

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**Fig. 2. Chemical cross-linking of Su g-Su e heterodimers.** A and B, upper panel, mitochondria (100 μg of protein) isolated from the wild type (WT) and the Δsu g null mutant strains expressing the Su gY112C (Y112C), or Su gY112CHA (Y112CHA) derivatives were treated with the chemical cross-linker DTNB (+) or were mock-treated and received Me$_2$SO (−), as described under "Experimental Procedures." After cross-linking and quenching of free DTNB, mitochondria were re-isolated by centrifugation, divided in half, and subjected to non-reducing SDS-PAGE and Western blotting. One of the resulting blots (A) was used to identify Su g and the Su gHA derivatives and their respective cross-linked adducts by decoration with Su g antisera or with HA-specific antisera, as indicated. Adducts with electrophoretic mobilities corresponding to Su e-Su g (e-g), Su e-Su gHA (e-gHA), and Su gHA-Su gHA (gHA-gHA) are indicated. Su e and cross-linked adducts were identified after decoration of the second blot with Su e-specific antisera (B, upper panel). Adducts with electrophoretic mobilities corresponding to Su e-Su e (e-e), Su e-Su g (e-g), Su e-Su gHA (e-gHA), and Su e-7 kDa (e-7kDa) are indicated. A protein that cross-reacts with the Su e antisemur (present also in the Su e null mutant (results not shown)) is indicated (*). B, lower panel, mitochondria (100 μg of protein) isolated from the wild-type (WT) and the Δsu g null mutant strains expressing the Su gY110CCHA (Y110CCHA) or Su gY112CHA (Y112CHA) derivatives were subjected to cross-linking with DTNB and further processed as described above in (B, upper panel). The resulting blot was decorated with Su e-specific antisera. The mobilities of the protein standards (kDa) are indicated.

**When Su g and Su e are expressed together in mitochondria, Su g becomes cross-linked to the Cys-modified Su g derivatives, represents Su e, a parallel DTNB-cross-linking experiment was immune-decorated with antibodies specific for Su e (Fig. 2B, upper panel). In addition to forming the previously described Su e-Su e homodimer of 22 kDa (10) and an 18-kDa adduct (see the legend of Fig. 4 for further discussion), Su e was also observed to form cross-linked adducts of 23- and 24-kDa size in the presence of Su gY112C and Su gY112CHA derivatives, respectively. These Su e-containing adducts were notably absent in the wild-type mitochondria, which contained the authentic, i.e. non-Cys-modified Su g protein. Taking together, the mass of the Su e-containing adducts, the fact that they occur only in the presence of the Cys-modified Su g, and the observed increase in size of the adduct when cross-linking was performed in the HA-tagged Su g mutant mitochondria allow us to conclude that the observed 23- and 24-kDa adducts represent Su g-Su e cross-linked partners. We conclude, therefore, that Su e and Su g can be found in close proximity to each other in the mitochondrial inner membrane. Interestingly, the Su e-Su g adducts were observed to form with a significantly greater efficiency than either the Su e-Su e homodimer or the putative Su g-Su g dimer.

Cross-linking of Su g to Su e was also observed using the second independent Su g Cys mutant, Su gY110C(HA) (Fig. 2B, lower panel). However, the efficiency of cross-linking of Su g-Su e appeared to be greater in the case of the Su gY112CHA derivative. The observed difference in the cross-linking efficiency between Su gY112CHA and Su gY112CHA may be accounted for by a positioning effect of the residues. In the folded structure of Su g, residue Val-110 may be positioned away from the Cys-27 residue of Su e, whereas the residue 112 may be physically closer to it.

**Mutations in the Conserved GXXXG Motif of Su g—As previously indicated, Su g contains a highly conserved GXXXG motif in its transmembrane segment. To address the importance of this motif for the stability of Su g and its molecular organization within the F$_{0}$-sector, a series of mutated Su g derivatives was constructed. First, the conserved Gly residues of the $^{103}$GXXXG$^{105}$ motif were individually replaced by bulkier hydrophobic residues, either Leu or Val. The resulting Su g mutants were cloned as HA-tagged proteins, Su gG101L(HA), Su gG101V(HA), Su gG105L(HA), and Su gG105V(HA). In addition, another Su g mutant was constructed whereby the GXXXG motif was disrupted by the insertion of an extra Ala residue between residues 103 and 104, i.e. conversion of GEIIG to GEIAIG, thus resulting in the creation of a HA-tagged Su gG103A104(HA) derivative.**

The resulting Su g GXXXG motif mutants were expressed in the Δsu g strain, and mitochondria were isolated and analyzed for the presence of the mutated Su g derivatives (Fig. 3, A and B). Immune-decoration with HA-specific antiserum indicated that the Su gG105V(HA) derivative accumulated in the mitochondria at levels similar to those of the wild-type Su gHA derivative, whereas the levels of Su gG105L(HA) were slightly lower (Fig. 3A). The reduced levels of the Su gG105L(HA) relative to the Su gG105V(HA) derivative may be due to the fact that a Leu residue is bulkier than Val and may, therefore, be less favorable for the stability of Su g. The steady state levels of the Su gG103A104(HA) protein and in particular, the Su gG101L(HA) and Su gG101V(HA) derivatives, were severely reduced when compared with the Su gHA levels, however (Fig. 3B).

Yeast strains harboring the Su gG101L(HA), Su gG101V(HA), and Su gG103A104(HA) derivatives, like the Δsu g parent strain, displayed an enhanced frequency of rho$^{-}$/rho$^{-}$ formation (Fig. 3C). The observed instability of the mtDNA is most likely a result of the reduced and, therefore, limiting levels of the Su g protein in these strains. Yeast cells harboring the Su...
gG105V, gG105L, and gG101L mutants, and in the absence of the Su g protein, i.e. in the Δsu g mitochondria (Fig. 4, lower panel). Significant cross-linking of Su e to a smaller protein of ~7 kDa, as evidenced by the generation of a 18-kDa Su e-reactive adduct, was observed in the absence of Su g (i.e. in the Δsu g mitochondria) and also in the Δsu g mitochondria bearing either of the Gly-105-mutated forms of Su g. Formation of this 18-kDa Su e-adduct was also observed in the wild-type and Su gY112C derivatives, albeit at significantly reduced levels (Fig. 4, lower panel). Although the identity of this Su e-interacting protein is currently unknown, the greatly enhanced formation of the Su e-18-kDa adduct indicates that the molecular environment of Su e is greatly altered in the absence of Su g or in the presence of the Gly-105-mutated derivatives of Su g.

**Conserved GXXXG Motif of Su g and ATP Synthase Dimerization**—Using the mild detergent digitonin, the ATP synthase complex can be solubilized from wild-type mitochondria membranes and resolved on a native electrophoresis gel as a dimeric complex (~1000 kDa) (6). The presence of Su g and Su e is required for the formation of detergent-stable ATP synthase dimers, as only monomeric complexes are observed after detergent solubilization of mitochondria from the Δsu g and Δsu e strains (6, 9, 15). We, therefore, analyzed the dimeric state of the ATP synthase complex from mitochondria harboring the mutations in the conserved Gly-105 residue of the Su g protein.
Isolated mitochondria were treated with increasing concentrations of the mild detergent digitonin, and the solubilized proteins were analyzed by clear native-PAGE. The assembly state of the ATP synthase complex was analyzed after Western blotting and immune-decoration with antibodies raised against the purified F$_1$-sector (Fig. 5, upper panel). In the absence of Su g, i.e. in the Δsu g mitochondria, the ATP synthase complex was solubilized as the monomeric size complex, indicating that the presence of Su g is required for the formation of detergent-stable ATP synthase dimers (Fig. 5, upper panel). The expression of the HA-tagged wild-type Su g protein, Su g HA, in the Δsu g strain fully restored the ability of the ATP synthase to form stable dimeric complexes (Fig. 5, upper panel). We conclude, therefore, that the addition of the HA tag to the C terminus of Su g did not adversely affect the function of Su g in this respect. Clear native-PAGE analysis of the detergent-solubilized ATP synthase from the mitochondria harboring the Su g105VHA or Su g105LHA derivatives indicated the presence of both dimeric and monomeric forms of the enzyme (Fig. 5, lower panel). The ATP synthase dimers formed in the presence of Gly-105-mutated Su g derivatives were not as stable as those supported by the wild-type Su g HA, as evidenced by the increased presence of monomeric ATP synthase particularly at the higher detergent concentrations. Moreover, ATP synthase monomers were observed in the G105L mutant relative to the G105V mutant, and this may reflect the reduction in the steady state levels of the Su g105LHA derivative relative to the Su g105VHA protein. Analysis of the assembly state of the ATP synthase in the mitochondria harboring the Su g101LHA, Su g101VHA, or Su g103A104HA derivatives indicated the presence of the monomeric form of the enzyme (results not shown). The significant reduction in the levels of dimeric complexes in this case simply may reflect the strongly reduced steady state levels of the Gly-101-mutated or Su g103A104HA derivatives relative to wild-type or the Gly-105-mutated derivatives.

**DISCUSSION**

To gain more insight into the importance and the role(s) of Su g of the yeast F$_1$F$_0$-ATP synthase, we used site-directed mutagenesis and a sulphydryl-specific cross-linking approach to map the molecular environment of Su g within the F$_0$-sector. Using the zero-length spacer arm cross-linker DTNB, we provide evidence here for the close proximity of the Su g and Su e proteins in the mitochondrial inner membrane. Moreover, our data support that Su g and Su e are positioned in the inner membrane in such a manner that their membrane anchor domains are physically close together. Specifically, we demonstrate that a cysteine positioned at residue 112 of Su g can be cross-linked to Cys-27 of Su e using DTNB. These residues are located in the intermembrane space and are in close proximity (−7 and 6 residues, respectively) to the membrane anchor regions of their respective proteins.

Our conclusion that the membrane anchor regions of Su g and Su e are physically close to each other is consistent with recent observations concerning the stabilization of Su g by Su e. The presence of Su e is required for the stability of Su g (6, 15, 16), and recent findings indicate that the membrane anchor region of Su e and/or the hydrophilic residues immediately C-terminal to it, are critical for the stability of Su g (15). Moreover, mutation in the conserved GXXXG motif in the membrane anchor region of Su g had a pronounced effect on the stability of Su g (16). This together with our findings in this work confirm that Su g and Su e and in particular their membrane anchor regions are in close physical proximity to each other within the F$_0$-sector of the ATP synthase complex.

The GXXXG motif in the transmembrane segment of Su g is highly conserved from lower eukaryotes, such as yeast, to higher eukaryotes, e.g. humans (20), indicating the potential importance of this motif for the function of Su g. GXXXG motifs can play essential roles in the homo- and heterodimerization of a number of membrane proteins, as they form the basis for helix-helix interactions, with the homodimerization of glycoporphin A being one of the best-characterized model proteins (21–24). Given that there are ~3.6 residues per α-helical turn, the conserved Gly residues of the motif would be arranged on the same face of the helix, and the number of three-spacer residues separating them is critical to preserve this arrangement. The small nature of the side chain of Gly is compatible with the formation of a close helix-helix association (21–24). Exchange of Gly residues for hydrophobic ones with bulkier side chains, such as Ala, Val, Leu, or Ile, interfere with the packing interface of neighboring helices and consequently have been shown to prevent the dimerization of model membrane proteins such as glycoporphin A and integrin α$_{IIb}$ (23, 31, 32). Similarly, the introduction of an extra residue between the Gly residues of this motif prevent helix-helix association of glycoporphin A, as the conserved Gly residues are no longer be arranged on the same face of the α-helix (21). Our analysis here demonstrates that the conserved GXXXG$_{105}$ motif in Su g plays an important role in the function and stability of Su g in the mitochondria. The disruption of the GXXXG motif by the insertion of an additional Ala residue after amino acid 103 had severe effects on the stability of Su g, as evidenced by the reduced steady state levels of the Su g103A104 derivative. Similarly, our data show that the mutation of the conserved Gly residues affects both the stability and molecular organization of Su g within the ATP synthase complex. In both the case of Gly-101 and Gly-105, substituting the Gly residue for a Leu residue was observed to be more deleterious than substituting with a Val residue. Leu has a bulkier side chain than Val and, therefore, may adversely affect the helix-packing capacity of Su g transmembrane region to a greater extent. Of the two conserved Gly residues (Gly-101 and Gly-105), our data support a more critical role of residue Gly-101, which relative to Gly-105, is located more toward the center of the transmembrane helix of Su g. Substitution of Gly-101 by a bulkier residue, Val, and in particular, Leu, was deleterious for the stability of Su g, whereas the mutation of Gly-105 did not have such a pronounced adverse affect. Although the steady state levels of Su g appeared...
not to be as significantly reduced by mutation of the Gly-105 residue, we observed that the function of Su g was clearly compromised in Su gG105VHA and Su gG105LHA derivatives. Specifically, we demonstrated that the Su g derivatives bearing a mutation in residue Gly-105 were less able to support the stable dimerization of the ATP synthase complex and that cells harboring this Su g mutant displayed a reduced capacity to prevent loss of the mtDNA when compared with the wild-type Su gHA protein. Furthermore, our cross-linking data indicate that mutation of Gly-105 to either a Val or Leu residue had a pronounced effect on the ability of Su g to influence the organization of Su e within the ATP synthase complex. In the absence of Su g, i.e., in the ∆su g mitochondria, we report here that Su e displays an increased ability to form homodimers and also to become cross-linked to an unknown protein of ~7 kDa. Mitochondria harboring Su gG105V,Y112C HA or Su gG105L,Y112C HA derivatives both displayed a similar alteration in the molecular organization of Su e, as was observed in the ∆su g mitochondria. Thus, although the mutation of Gly-105 of Su g did not have such a pronounced effect on the stability of Su g as the Gly-101 mutation, it clearly compromised the function of Su g, in particular its ability to stabilize the mtDNA and to modulate the environment of Su e. All of these observations together allow us to conclude that the conserved GXXXG motif plays a critical role in the stability of Su g and its ability to modulate the molecular organization of components of the ATP synthase in particular Su e. We propose, therefore, that the GXXXG motif is required to support the interaction of Su g with another membrane protein. As discussed below, one possible candidate for this interacting protein would be Su e.

Su e also has a conserved GXXXG motif in its membrane anchor segment, and the presence of Su e is required for the stability of Su g (15, 16). Velours and co-workers (16) have previously shown that mutation of the conserved Gly residues of the GXXXG motif in Su e or the disruption of this motif by the insertion of an extra hydrophobic residue results in the inability of Su e to ensure the stabilization of Su g. These comments, together with our findings reported here, allow us to predict that the transmembrane region of Su g, and in particular the conserved GXXXG motif, could be the foundation for the observed Su g-Su e interaction. In turn, this interaction with Su e would ensure the stability of Su g in the membrane. Contrary to this notion, however, we demonstrate here that an intact GXXXG motif in Su g is not required to support an interaction between Su g and Su e. The exchange of Gly-105 for Val or Leu residues did not have a measurable effect on the ability of Su g to become cross-linked to Su e despite the fact that this mutation compromises a number of functions of Su g as outlined above. If the GXXXG motif of Su g operates as a dimerization motif and in a similar manner as in glycoporphin A or integrin β₆ (33, 31, 32), mutation of Gly-105 to Leu or Val would have disrupted helix-helix packing and, hence, the ability of Su g to interact with Su e. In addition, our data would support that the GXXXG motif is not the basis for the possible Su g-Su g homo-dimerization we observed. An increase in the level of Su g-Su g interaction was observed in the case of the Su g derivative bearing the Gly-105 mutations.

Hence, we conclude that an intact GXXXG motif in Su g is not essential for the observed Su g-Su e interaction or the putative Su g-Su g homodimerization. Although it remains possible that the GXXXG motif is somehow involved in the interaction of the transmembrane segments of Su g with Su e, it does not appear act as a canonical GXXXG helix-helix interaction motif, as has been described for glycoporphin A. We conclude, therefore, that the GXXXG motif of Su g may not be the basis (or sole basis) for the Su g-Su e interaction. While interacting together in the membrane, it is possible that Su g and Su e may also interact with yet another protein and in a manner that requires the GXXXG motifs of Su g and Su e. In the absence of Su g (or in mitochondria bearing the Gly-105-mutated Su g derivatives), our cross-linking data that indicate a closer association between Su e and an unknown protein of ~7 kDa is favored. The identity of this 7-kDa protein is currently unknown. The mass of this protein is comparable with that of two other known Fₒ-subunits, Atp8 and Atp9. Because Atp8 does not contain a Cys residue, we can exclude it as a candidate, as the cross-linking to Su e was observed with the sulf-hydryl-specific cross-linking agent DTNB. In addition, immune-decoration of Western blots of cross-linking experiments with Atp9 antiserum have not provided support for the possibility that the 7-kDa candidate may represent Atp9. We, therefore, are adopting other approaches to purify and identify this 7-kDa interacting protein. We propose by interacting together (and possibly with the 7-kDa protein also), Su g and Su e exert functions that are distinct from their ability to stabilize the ATP synthase dimers and by doing so ensure the maintenance of a tightly coupled ATP synthase, as suggested by the reduced growth and the loss of mtDNA phenotypes observed with the su e and su g null mutant strains. Understanding the molecular organization of Su g and Su e within the Fₒ-sector represents an essential step toward fully appreciating the significance of these conserved proteins.

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The Yeast $F_1F_0$-ATP Synthase: ANALYSIS OF THE MOLECULAR ORGANIZATION OF SUBUNIT $\gamma$ AND THE IMPORTANCE OF A CONSERVED GXXXG MOTIF
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