The Modification of the Conserved GXXXG Motif of the Membrane-spanning Segment of Subunit g Destabilizes the Supramolecular Species of Yeast ATP Synthase*

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The supernumerary subunit g is found in all mitochondrial ATP synthases. Most of the conserved amino acid residues are present in the membrane-C-terminal part of the protein that contains a dimerization motif GXXXG. In yeast, alteration of this motif leads to the loss of subunit g and of supramolecular structures of the ATP synthase with concomitant appearance of anomalous mitochondrial morphologies. Disulfide bond formation involving an engineered cysteine in position 109 of subunit g and the endogenous cysteine 28 of subunit e promoted g + g, e + g, and e + e adducts, thus revealing the proximity in the mitochondrial membrane of several subunits e and g. Disulfide bond formation between two subunits g in mitochondria increased the stability of an oligomeric structure of the ATP synthase in digitonin extracts. These data suggest the participation of the dimerization motif of subunit g in the formation of supramolecular structures and is in favor of the existence of ATP synthase associations, in the inner mitochondrial membrane, whose masses are higher than those of ATP synthase dimers.

FₐFₐ-ATP synthase is a molecular rotary motor that is responsible for aerobic synthesis of ATP. It exhibits a headpiece (catalytic sector), a base piece (membrane sector), and two connecting stalks. Sector F₁, containing the headpiece is a water-soluble unit that retains the ability to hydrolyze ATP when in soluble form. F₀ is embedded in the membrane and is mainly composed of hydrophobic subunits forming a specific proton-conducting pathway. When the F₁ and F₀ sectors are coupled, the enzyme functions as a reversible H⁺-transporting ATPase or ATP synthase (1–4). The two connecting stalks are made of components from F₁ and F₀. The central stalk is a part of the rotor of the enzyme and the second stalk, which is part of the stator, connects F₁ and hydrophobic membranous components of the enzyme probably via a flexible region (1). High resolution x-ray crystallographic data have led to solving the structure of F₁ from different sources (5–9) and the F₁-c₁₀-ring from Saccharomyces cerevisiae (9).

The mitochondrial F₀ of mammals is composed of 10 different subunits (10), all identified in the S. cerevisiae enzyme (11–13). Some of these subunits are not required for ATP synthase function, but are involved in the dimerization/oligomerization of the mitochondrial ATP synthase (13–15). For example, null mutants of subunits g and e abolish the ability of ATP synthase to make supramolecular structures. These subunits are small hydrophobic proteins that have only one spanning segment with a N terminus inside the matrix, and the C terminus in the intermembrane space (12, 16) with a membrane-spanning segment probably located at the interface between two ATP synthase monomers (16).

The subunits g and e have a conserved putative dimerization GXXXG motif located in the membrane-spanning segment. In subunit e, its alteration led to the loss of subunit g and the loss of dimeric and oligomeric forms of the yeast ATP synthase. The presence of a cysteine residue (Cys²⁸) placed after the membrane domain of subunit e made it possible to establish, by cross-linking experiments, that two subunits e are close to each other in the membrane. This disulfide bond was shown to significantly stabilize ATP synthase dimerization/oligomerization in intact mitochondria (17). The subunit e also contains a putative coiled-coil region in its C-terminal part, which is involved in the stabilization of the dimeric forms of the detergent-solubilized ATP synthase complexes (18). The study of subunits g and e is also important because the dimerization/oligomerization process of ATP synthase complex is linked to cristae biogenesis and mitochondrial morphology (13, 15).

The most highly studied, and apparently widespread, mode of helix-helix association is mediated by the so-called GXXXG motif, which is known to act as a universal scaffold for the assembly of two transmembrane helices (19). The GXXXG is a motif where two glycine residues are separated by any three amino acids on a helical framework. This arrangement of glycine residues allows the close approach of interacting helices, whereupon extensive packing interactions take place between pairs of surrounding residues. Despite the high occurrence of the GXXXG motif in transmembrane helices, the transmembrane peptide of glycoporphin A is the only dimer mediated by a GXXXG motif for which the structure has been determined to high resolution (20). Thus, it is not clear whether alternate structures for transmembrane dimers exist. Moreover, it is not known how residues surrounding GXXXG motifs “tailor” the affinity of their helix-helix interactions for required structural and functional purposes (21).

Here, site-specific mutagenesis was used to modify the membranous domain, and especially the GXXXG domain of subunit g of the ATP synthase, to determine its interaction with differ-
ent subunits of the complex and its participation in the edification of supramolecular ATP synthase species.

**EXPERIMENTAL PROCEDURES**

**Materials**—Digitonin was from Sigma. Oligonucleotides were purchased from MWG-BIOTECH. All other reagents were of reagent grade quality.

**Yeast Strains and Nucleic Acid Techniques**—The S. cerevisiae strain D273-10B/A1/1U (MATa, met6, ura3, his3) was the wild-type strain. The yeast mutants with a point mutation were named as (name of the subunit) (one-letter code of wild-type residue) (residue number) (mutant residue) (i.e., eC228S). The null mutant in the ATP20 gene (Δg) was constructed by PCR-based mutagenesis, and the kan" gene was removed. The gene ATP20 encoding subunit g was obtained by PCR amplification of genomic DNA and the resulting 1178-bp EcoRI-XhoI DNA fragment was cloned in the shuttle vector pRS313. A 1586-bp Pru1-EcoRV DNA fragment containing kan" was isolated from the pUG6 vector and inserted into the EcoRV site in the 3' region of the ATP20 gene. The mutations gQ93A, gY98A, gG101L, gG105L, and gC75S/L109C were introduced by a PCR mutagenesis procedure into the resulting vector. The strains containing modified versions of subunit g were obtained by selection of the resistance for a chromosomal locus of a 2777-bp EcoRI-Apal DNA fragment, bearing the mutated versions of the ATP20 gene, in the Δg strain, and were selected for their resistance to genetin. The strain containing the subunit gC75S/L109C was constructed according to the following strategy. Two complementary oligonucleotides, 5'-TATATAACATCACCAACACGCAACCTAGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT GG
positions 93, 98, 102, and 106, which display E/Q, F/Y, E/Q, and K/R residues, respectively are semiconservative. The transmembrane segment of subunit g displays a dimerization motif, GXXG, found in glycolphorin A (19) at position G101L (residues 101 to 105). The full conservation of the glycine residues suggested their involvement in a transmembrane helix-helix interaction. To address whether the conserved amino acid residues have a role in a dimerization process of the ATP synthase, several mutants were constructed. Mitochondria were prepared from the truncated mutants gV106stop, gR106stop, and gY112stop, from the gG101L and gG105L strains where the small residue was replaced by a large residue, and from mutant g102A having an alanine residue inserted after Gly101 to disrupt a helix-helix packing interface. The phenotypes and ATPase activities are reported in Table I.

With the exception of the mutant gY112stop all other mutants cited above displayed an increase in the doubling time with lactate as carbon source. In addition, a spontaneous conversion into rho− cells was observed. As reported previously (15, 17), there is a correlation between the increase in the spontaneous rho− cell conversion (rho− cells that are devoid of oxidative phosphorylation are unable to grow with lactate as carbon source) and the increase in the generation time of mutant strains. It is also possible to correlate the increase in the spontaneous rho− cell conversion with the increase in the insensitivity of the mitochondrial ATPase activity of the five mutants toward oligomycin (an inhibitor of membranous domain of the mitochondrial ATP synthase) under the experimental conditions used (pH 8.4 and Triton X-100) (Table I).

The wild-type strain was D273–10B/A/H/U. Yeast cells were grown on complete medium containing 2% lactate as carbon source. The doubling time was calculated for a 10-h period during the logarithmic growing phase. The rho− cell production in cultures was measured on glycerol plates supplemented with 0.1% glucose. ATPase activities and sensitivity to oligomycin (6 μg/ml) were measured in the presence of Triton X-100 to remove the F1 inhibitor.

### Table I

**Phenotypic analyses of the yeast strains used**

The wild-type strain was D273–10B/A/H/U. Yeast cells were grown on complete medium containing 2% lactate as carbon source. The doubling time was calculated for a 10-h period during the logarithmic growing phase. The rho− cell production in cultures was measured on glycerol plates supplemented with 0.1% glucose. ATPase activities and sensitivity to oligomycin (6 μg/ml) were measured in the presence of Triton X-100 to remove the F1 inhibitor.

| Strains | Doubling time | % of rho− cells in cultures | ATPase activities | Inhibition |
|---------|---------------|-----------------------------|-------------------|-----------|
|         | min | % | Control | + Oligomycin | μmol of P/min | mg of protein | % |
| Wild type | 182 | 1 | 6.68 ± 0.29 | 0.51 ± 0.10 | 92 |
| gC75S/L109C | 168 | 1 | 6.78 ± 0.03 | 0.53 ± 0.05 | 92 |
| eC28S/gC75S/L109C | 170 | 2 | 6.66 ± 0.19 | 0.60 ± 0.06 | 91 |
| gQ93A | 244 | 25 | 6.49 ± 0.30 | 2.02 ± 0.03 | 69 |
| gQ38E | 186 | 1 | 6.60 ± 0.09 | 0.51 ± 0.09 | 92 |
| gY98A | 364 | 37 | 6.98 ± 0.06 | 2.36 ± 0.06 | 66 |
| gV106stop | 238 | 28 | 6.98 ± 0.05 | 2.83 ± 0.02 | 59 |
| gG101L | 283 | 29 | 6.67 ± 0.05 | 2.58 ± 0.08 | 61 |
| g102A | 306 | 30 | 6.72 ± 0.37 | 2.78 ± 0.08 | 59 |
| gG105L | 283 | 27 | 6.87 ± 0.05 | 2.78 ± 0.02 | 59 |
| gR106stop | 250 | 17 | 6.03 ± 0.42 | 2.47 ± 0.09 | 59 |
| gY112stop | 178 | 1 | 6.56 ± 0.02 | 0.45 ± 0.04 | 93 |

The Dimerization of Subunit g Are Essential for the Presence of Subunit g in the Mitochondrial Membrane—Subunit g is an unstable protein that disappears upon alteration of either subunits e or subunit 4 (b) (14). As a consequence, the presence of subunit g in mutant mitochondria was examined by Western blot analysis. The blots were probed with polyclonal antibodies raised against subunits g or e. Subunit i, which is a component of the yeast F1 subunit, was used as control. The gQ93A, gY98A, gG101L, g102A, and gG105L mutant mitochondria were fully devoid of subunit g (Fig. 2), and subunit e was found principally as a dimer, resulting from the oxidation of Cys28, an observation that has been already reported in mutants devoid of subunit g (29). In addition, an unidentified product involving subunit e was observed in the molecular mass range of 17 kDa. A small amount of subunit g was found in gV106stop and gR106stop mitochondria, thus showing that a subunit g at least 106 amino acids long is required for its presence in the mitochondrial membrane. However, the truncated mutant gY112stop has no alterations in physiological parameters (Table I). It shows a normal amount of the modified subunit g as revealed by Western blot (not shown), indicating that conserved residue Tyr112 is not involved in a stabilizing function. Whereas an alanine residue could not replace Gln93, a glutamate residue (gQ93E) altered neither the generation time (Table I) nor the presence of subunit g in the mitochondrial membrane. These data are in agreement with the presence of a glutamate residue at this position in other subunits g (Fig. 1A).

It has been previously reported that the absence of subunit g in the null mutant in the ATP20 gene leads to the loss of supramolecular structures of the ATP synthase (13). Therefore, the presence of supramolecular species of the ATP synthase in the mitochondrial diconitin extracts of gV106stop and gR106stop mutants was examined by BN-PAGE. The diconitin extracts were loaded on a 3–13% acrylamide slab gel, and the mitochondrial complexes were separated under native conditions. The gel was incubated with ATP-Mg2+ and Pb2+ to reveal the ATPase activity (Fig. 3). As reported previously, the wild-type diconitin extracts contained the dimeric and oligomeric forms of the enzyme that were destabilized upon increasing the diconitin-to-protein ratio (13, 29). The mitochondrial diconitin extracts of mutant strains did not display any oligomeric form of the ATP synthase. Whatever the diconitin-to-protein ratio used, the monomeric form of the enzyme was
The involvement of the GXXG motif of subunit g in the formation of a homodimer was investigated. Because the relative molecular masses of subunits e and g are very similar, the C75S/L109C strain by complementation with a pRS316 shuttle vector bearing the gene encoding gC75S/L109C(His)_4. For the last mutant, Western blot analysis of CuCl2-treated mitochondria displayed three bands in the 26-kDa region, which were gC75S/L109C plus gC75S/L109C(His)4. To demonstrate that the 26-kDa band corresponded to a homodimer of subunit g resulting from the formation of a disulfide bond between the two subunits e and g, the two following strains were constructed: strain eC28S/gC75S/L109C(His)4 contained a (His)_4 tag in the C-terminal part of subunit g. The second mutant was constructed from the eC28S/gC75S/L109C strain by complementation with a pRS316 shuttle vector bearing the gene encoding gC75S/L109C(His)_4. For the last mutant, Western blot analysis of CuCl2-treated mitochondria displayed three bands in the 26-kDa region, which were gC75S/L109C plus gC75S/L109C(His)_4. To demonstrate that the 26-kDa band corresponded to a homodimer of subunit g resulting from the formation of a disulfide bond between the two subunits e and g, the two following strains were constructed: strain eC28S/gC75S/L109C(His)_4 contained a (His)_4 tag in the C-terminal part of subunit g. The second mutant was constructed from the eC28S/gC75S/L109C strain by complementation with a pRS316 shuttle vector bearing the gene encoding gC75S/L109C(His)_4. For the last mutant, Western blot analysis of CuCl2-treated mitochondria displayed three bands in the 26-kDa region, which were gC75S/L109C plus gC75S/L109C(His)_4. To demonstrate that the 26-kDa band corresponded to a homodimer of subunit g resulting from the formation of a disulfide bond between the two subunits e and g, the two following strains were constructed: strain eC28S/gC75S/L109C(His)_4 contained a (His)_4 tag in the C-terminal part of subunit g. The second mutant was constructed from the eC28S/gC75S/L109C strain by complementation with a pRS316 shuttle vector bearing the gene encoding gC75S/L109C(His)_4. For the last mutant, Western blot analysis of CuCl2-treated mitochondria displayed three bands in the 26-kDa region, which were gC75S/L109C plus gC75S/L109C(His)_4. To demonstrate that the 26-kDa band corresponded to a homodimer of subunit g resulting from the formation of a disulfide bond between the two subunits e and g, the two following strains were constructed: strain eC28S/gC75S/L109C(His)_4 contained a (His)_4 tag in the C-terminal part of subunit g. The second mutant was constructed from the eC28S/gC75S/L109C strain by complementation with a pRS316 shuttle vector bearing the gene encoding gC75S/L109C(His)_4. For the last mutant, Western blot analysis of CuCl2-treated mitochondria displayed three bands in the 26-kDa region, which were gC75S/L109C plus gC75S/L109C(His)_4. To demonstrate that the 26-kDa band corresponded to a homodimer of subunit g resulting from the formation of a disulfide bond between the two
The Dimerization of Subunit \( g \)

FIG. 6. Oxidation promotes the homodimerization of subunits \( g \) and \( e \) and the heterodimerization of subunits \( g \) and \( e \). Mitochondria (mit.) isolated from \( gC75S/L109C(His)_6 \) cells and the mitochondrial digitonin extracts obtained with a digitonin-to-protein ratio of 0.75 g/g (ext.) were incubated with different concentrations of CuCl\(_2\), as described under “Experimental Procedures.” In the absence of CuCl\(_2\), incubation was performed in the presence of NEM and EDTA. After dissociation of samples, aliquots (50 μg of protein) were analyzed by Western blot. The blots were incubated with polyclonal antibodies raised against subunits \( g \) and \( i \) (A), then membranes were washed and probed again with antibodies against subunits \( e \) and \( i \) (B). \( g^* = gC75S/L109C(His)_6 \) plus \( gC75S/L109C(His)_6 \) heterodimer, and a dimer of subunit \( e \) in mitochondrial membranes, thus indicating that \( e \) and \( g \) subunits are neighbors in the yeast inner mitochondrial membrane as in bovine mitochondria (16). It appears also that the heterodimer of subunits \( e \) and \( g \) is more intense than the dimer of subunits \( e \), probably indicating a smaller distance between the Cys\( ^{108} \) of subunit \( g \) and the Cys\( ^{28} \) of subunit \( e \) than between two Cys\( ^{28} \). Another interpretation is that there are different accessibility to the antibodies against subunit \( e \) on heterodimers of subunits \( e \) and \( g \) and homodimers of subunits \( e \). On the other hand, the \( g \) plus \( g \) and \( e \) plus \( e \) cross-linked products displayed similar intensities. When digitonin extracts were incubated with CuCl\(_2\), the \( e \)- and \( gC75S/L109C(His)_6 \) subunits were cross-linked, whereas the formation of a disulfide bond between two subunits \( e \) and between two subunits \( g \) were decreased, thus showing different behavior of subunits \( g \) and \( e \) in mitochondrial membranes and in detergent extracts, but also the existence of digitonin-stable homodimers of subunits \( g \) and subunits \( e \).

The Disulfide Bond Formation between Two Subunits \( g \) Stabilizes an Oligomeric Form of the Yeast ATP Synthase—In a previous paper (17), it has been reported that the formation of a disulfide bridge between two subunits \( e \) via the Cys\( ^{28} \) by oxidation of mitochondrial membranes leads to the stabilization of an oligomeric form of the ATP synthase in digitonin extracts. Similar experiments involving the mutation g109C were performed. Mitochondria and digitonin extracts of the \( eC28S/gC75S/L109C \) strain were oxidized in the presence of CuCl\(_2\). Mitochondria were solubilized with digitonin, and detergent extracts were submitted to a BN-PAGE analysis. The digitonin-to-protein ratios of 0.75 and 2 g/g were chosen to extract the membranous proteins. With such ratios, \( F_iF_o \) oligomers, \( F_iF_o \) dimers, \( F_iF_o \) monomer, and \( F_i \) were clearly observed. BN-PAGE analysis revealed the presence of an oligomeric form of the ATP synthase in digitonin extracts of CuCl\(_2\)-treated mitochondria migrating at an acrylamide concentration of 4.8% (Fig. 7A), despite a digitonin-to-protein ratio of 2 g/g, i.e. conditions that fully destabilize the oligomeric forms of the yeast ATP synthase. On the other hand, oxidation of \( eC28S/gC75S/L109C \) digitonin extract (digitonin-to-protein ratio of 2 g/g) did not promote the oligomer formation (Fig. 7B).

The Homodimers of Subunits \( g \) and \( e \) Are Found Only with the Oligomeric Forms of the Yeast ATP Synthase, whereas the Heterodimers of Subunits \( g \) and \( e \) Are Associated with the Dimeric Forms of ATP Synthase—To investigate the positions of different homo- and heterodimers of subunits \( e \) and \( g \) in the supramolecular species of ATP synthase, BN-PAGE analyses were performed with mitochondrial digitonin extracts obtained with a digitonin-to-protein ratio of 0.75 g/g. Under these conditions, only dimeric and oligomeric forms of ATP synthase were found (17). The \( gC75S/L109C(His)_6 \) mutant was used to increase the mass of the different adducts. After copper treatment of mitochondria and BN-PAGE analysis of digitonin extracts, gel slices were cut and submitted to SDS-gel electrophoresis in the second dimension. The proteins were transferred onto a nitrocellulose membrane that was probed with polyclonal antibodies against subunit \( g \). Polyclonal antibodies directed against subunit \( i \) were also used as control to detect the position of the different forms of ATP synthases, because subunit \( i \) is strongly associated with the enzyme at digitonin-to-protein ratio of 0.75 g/g (17, 31). Membranes were stripped and probed again with antibodies against subunits \( e \) and \( i \). When copper-treated \( gC75S/L109C(His)_6 \) mitochondria were solubilized with digitonin, subunits \( g \), \( e \), and \( i \) were mainly associated with ATP synthases, and co-migrated with the dimeric and oligomeric forms during native electrophoresis (Fig. 8, A and B). However, the subunit \( g \) and subunit \( e \) homodimers were found in the oligomeric form of the ATP synthase not in the dimeric form. The heterodimers of subunit \( g \) and \( e \) were observed mainly in the dimeric form but also in the oligomeric form of the ATP synthase (Fig. 8, A and B). However, it should be noted that a large amount of the heterodimer of subunits \( g \) and \( e \) was dissociated from the enzyme because it migrated in the front of the BN-PAGE, thus indicating that severe constraints were produced by the cross-link of \( e \) and \( g \) subunits that led to the dissociation of the \( e + g \) adduct from the supramolecular species.

DISCUSSION

The purpose of the present paper was to investigate the role of subunit \( g \) in the dimerization/oligomerization process of ATP synthase and to look for the molecular determinants leading to the association of ATP synthases in the inner mitochondrial membrane. Subunits \( g \) and \( e \) are two supernumerary subunits that are loosely bound to the ATP synthase because they are not present in the monomeric form of the yeast enzyme (15). They are not involved in ATP synthesis but most probably in the biogenesis and folding of cristae, because in their absence...
mitochondria display onion-like structures (15, 32) at the same time as the supramolecular structures of the ATP synthase are lost. Like subunit e, subunit g has a membranous GXXXG dimerization motif that could be the basis of the dimerization/oligomerization of mitochondrial ATP synthases.

The involvement of the membranous GXXXG motif in the dimerization/oligomerization of mitochondrial ATP synthases—The involvement of the GXXXG motif of subunit e in the formation of supramolecular species of ATP synthase has recently been shown (17). Although, subunit g is closely related to subunit e, neither its dimerization nor its involvement in the architecture of supramolecular species of the ATP synthase has been reported so far. The GXXXG motif provides a basic scaffold responsible for mediating transmembrane helix-helix interactions. Indeed, early studies on glycoporphin A have shown that the central GXXXG portion was the most crucial part of the interaction motif, as judged by its hypersensitivity to mutation, and its ability to singularly mediate the assembly of otherwise monomeric sequences. Although biased to this right-handed crossing motif, it was found that 80% of the high-affinity isolates contained the GXXXG motif (21).

Replacement of the first and last amino acid residues of the GXXXG motif of subunit g by leucine residues, the insertion of an alanine residue in the putative dimerization motif, and the truncation of subunit g after residues 99 and 105 led to the loss of subunit g, with the following consequences: a spontaneous homodimerization of subunit e by its unique cysteine residue, which was found loosely or not associated to the yeast ATP synthase in mutants devoid of subunit g (17), and a concomitant loss of supramolecular species of ATP synthase, highlighting the importance of the GXXXG motif and surrounding residues in the stability of subunit g in the mitochondrial membranes. We already know that this stability is dependent on the presence of subunit e and the first membrane-spanning segment of subunit d. To explore the environment of subunit g, the gC75S/L109C mutant was constructed, thus generating a target located in the intermembrane space. Incubation of mitochondrial membranes with copper led to a homodimer of subunit g and a heterodimer of subunits e and g resulting from disulfide bond formation between two gL109C and between gL109C and eC28, respectively. In addition, subunit e homodimer was found as described previously (17). Thus, these data reveal the proximity in the mitochondrial membrane of several subunits e and g that likely interact. An interaction domain between subunits e and g mediated by their respective GXXXG motifs was unlikely because the GXXXG motif of subunit e is located in the middle of the spanning segment (17) and that of subunit g in the C-terminal part of the spanning segment, the formation of the e + g adduct by oxidation being the consequence of the proximity of the respective cysteine residues. The dimer formation of subunits g by the Cys109 located on the same face as the GXXXG motif more likely indicates that this motif constitutes an interaction domain between two subunits g in the inner mitochondrial membrane. However, the amount of the g – g adduct was highly decreased in copper-treated digitonin extracts, whereas the amount of the e – g adduct was not. This indicates that the interface between subunits e and g was kept in the digitonin extracts, which contain supramolecular species of ATP synthase, whereas that between two subunits g and between two subunits e was not (Fig. 6). We hypothesize that the e – g adduct is the result of an intramolecular cross-linking and that the adduct between two subunits e and between two subunits g is the result of a cross-linking between ATP synthase dimers because the e – e and g – g adducts were found mainly associated with the oligomeric forms of ATP synthase (Fig. 7).

Oligomeric forms of mitochondrial ATP synthase—Whether oligomeric forms of the ATP synthase exist in the inner mitochondrial membrane is still a matter of discussion. In yeast, oligomeric forms of ATP synthase have been found in mitochondrial digitonin extracts obtained with digitonin-to-protein ratios of 0.75 g/g, but they were absent at higher ratios. However, the formation of disulfide bonds either between subunits e via Cys28 in wild type mitochondria (17) or between subunits g via the L109C residues promoted an oligomeric form of ATP synthase that was stabilized in digitonin extracts and which migrated at an acrylamide concentration of 4.8%, thus corresponding to a tetrameric form of the enzyme (15). The association by oxidation of ATP synthases in supramolecular structures higher than dimeric forms could result from the Brownian lateral diffusion of proteins in the inner mitochondrial membrane. However, (i) the experiments were performed at 4 °C to decrease the diffusion, (ii) no other cross-links between the ATP synthase and other mitochondrial complexes have been identified so far, (iii) the oligomeric structures of ATP synthase exist without cross-linking in mitochondrial digitonin extracts, and (iv) cross-linked oligomeric arrangements of ATP synthase in yeast cells have been obtained during periods of respiratory growth (33). Taken together these data are in favor of the existence in the inner mitochondrial membrane of associations of ATP synthases whose masses are higher than those of ATP synthase dimers. Such oligomeric forms imply the existence of two different interfaces between ATP synthase monomers. On the basis of cross-linking data on mitochondrial membranes, it was proposed that two subunits d (subunit b) belonging to two neighboring ATP synthases participate at one interface (34). Recent data indicate that this interface more likely involves the peripheral stalk of the ATP synthase because homodimers of the yeast F1b (subunit b) have been obtained by cross-linking (35). Moreover, a recent report showed that dimerization of the chloroplast ATP synthase of...
Clamydonomonas reinhardtii (an enzyme devoid of subunits e and g) has been described for many protein-protein interactions, where a mutated sequence could be retained if compensatory mutations that preserve the interaction occur in its interacting partners (40). Experiments are underway to define the interaction domains between subunits e and g.

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