The Peripheral Stalk Participates in the Yeast ATP Synthase Dimerization Independently of e and g Subunits †

ARTICLE *in* BIOCHEMISTRY · JUNE 2006

Impact Factor: 3.02 · DOI: 10.1021/bi0601407 · Source: PubMed

5 AUTHORS, INCLUDING:

Remi Fronzes
Institut Pasteur
25 PUBLICATIONS **991** CITATIONS
SEE PROFILE

Brèthes Daniel
French National Centre for Scientific Research
69 PUBLICATIONS **1,575** CITATIONS
SEE PROFILE

The Peripheral Stalk Participates in the Yeast ATP Synthase Dimerization Independently of e and g Subunits†

Rémi Fronzes,‡ Théodore Weimann, Jacques Vaillier, Jean Velours, and Daniel Brèthes*

Institut de Biochimie et Génétique Cellulaires du Centre National de la Recherche Scientifique, UMR5095, Université Victor Segalen Bordeaux 2, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France

Received January 23, 2006; Revised Manuscript Received April 5, 2006

ABSTRACT: It is now clearly established that dimerization of the F_1F_o ATP synthase takes place in the mitochondrial inner membrane. Interestingly, oligomerization of this enzyme seems to be involved in cristae morphogenesis. As they were able to form homodimers, subunits 4, e, and g have been proposed as potential ATP synthase dimerization subunits. In this paper, we provide evidence that subunit h, a peripheral stalk component, is located either at or near the ATP synthase dimerization interface. Subunit h homodimers were formed in mitochondria and were found to be associated to ATP synthase dimers. Moreover, homodimerization of subunit h and of subunit i turned out to be independent of subunits e and g, confirming the existence of an ATP synthase dimer in the mitochondrial inner membrane in the absence of subunits e and g. For the first time, this dimer has been observed by BN-PAGE. Finally, from these results we are now able to update our model for the supramolecular organization of the ATP synthase in the membrane and propose a role for subunits e and g, which stabilize the ATP synthase dimers and are involved in the oligomerization of the complex.

The F_1F_o ATP synthase of *Saccharomyces cerevisiae* is a 600 kDa enzymatic complex of the mitochondrial inner membrane. This reversible enzyme couples the use of the H^+ electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) generated by the respiratory chain to ATP synthesis and can be described as a molecular rotary motor in two parts, a rotor and a stator. The rotor, which is driven by the proton flux through the inner membrane, is composed of a 10 copy oligomer of subunit 9 and of subunits γ, δ, and ϵ. The stator comprises 9 major subunits, α, β, OSCP, 4, d, h, f, 8, and 6. Subunit β holds the catalytic sites and is organized with subunit α in a soluble $(\alpha\beta)_3$ hexamer. A peripheral stalk comprising subunits 4, d, h, f, 8, and OSCP links this catalytic hexamer to subunit 6 which, in interaction with the subunit 9 oligomer, forms a specific proton pathway within the membrane (*1*). According to the binding change mechanism model (*2*), the affinity change for substrates and products at catalytic sites is coupled to proton transport by the rotation of the γ subunit inside the $(\alpha\beta)_3$ hexamer. Historically, the ATP synthase complex has been separated into two different parts: (i) the yeast F_1 water-soluble sector, which is composed of the $(\alpha\beta)_3$ hexamer, γ, δ, and ϵ subunits, retains the ability to hydrolyze ATP in solution; (ii) the F_o sector, which is embedded in the inner membrane, is composed of both membrane (9, 6, 4, 8, and f) and soluble (OSCP, h, and d) subunits. ATP synthesis occurs only when the F_1 is bound to the F_o sector. In addition, two stalks have also been described. The central stalk is composed of subunits γ, δ, and ϵ while subunits 4, f, h, d, and OSCP form the peripheral stalk. This topological organization has been described in several organisms from bacteria to mammals. Whereas the F_1F_o ATP synthases of *Escherichia coli* and yeast share similar properties (*3*, *4*), the number of proteins constituting the bacterial stator is different. Indeed, yeast subunits 8, f, d, and h do not have any homologue in bacteria. In *E. coli*, the peripheral stalk is composed of only four proteins: two subunits b (homologue to yeast subunit 4) associated to subunit δ (homologue to yeast subunit OSCP) and subunit a (homologue to yeast subunit 6). In 1998, Spannagel et al. (*5*) showed that only one subunit 4 is present per eukaryotic ATP synthase complex. In yeast, the additional subunits, 8, f, d, and h, might compensate the absence of the second subunit b present in the bacterial peripheral stalk (*6*). Subunits e, g, i, and k are supernumerary subunits of the complex; i.e., these subunits are not essential for cellular growth on nonfermentable sources (*7*).

In 1998, Arnold et al. showed the presence of ATP synthase dimers in both Triton X-100 and digitonin extracts of wild-type yeast mitochondrial inner membranes using blue native polyacrylamide gel electrophoresis (BN-PAGE)[1] (*7*). The dimerization process was subsequently proposed to require the two supernumerary subunits e and g (*7*, *8*). In addition, proximity between two subunits 4 has been shown by the formation of disulfide bridges in the mitochondrial

† This work was supported by grants from the CNRS (AC Dynamique et Réactivité des Assemblages Biologiques and UMR5095), the Université Victor Ségalen Bordeaux 2, and the Conseil Régional d'Aquitaine. R.F. and T.W. held a research grant from the Ministère de la Recherche et la Technologie.

* Address correspondence to this author. Tel: +33-(0)5-56999047. Fax: +33-(0)5-56999051. E-mail: daniel.brethes@ibgc.u-bordeaux2.fr.

‡ Present address: School of Crystallography, UCL/Birkbeck College, Malet St., London WC1E 7HX, U.K.

[1] Abbreviations: SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BN-PAGE, blue native polyacrylamide gel electrophoresis; Ni-NTA, nickel nitrilotriacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

10.1021/bi0601407 CCC: $33.50
Published on Web 05/05/2006

inner membrane (*5*) and, very recently, in vivo by FRET (*9*). Since only one subunit 4 is present per ATP synthase, subunit 4 homodimerization has been proposed to involve two ATP synthase monomers (*5*). Since, in the absence of subunits e and g, the proximity between two subunits 4 still exists, as shown either by chemical cross-linking (*10*) or by FRET (*9*), it was suggested that two independent ATP synthase dimerization interfaces might exist, i.e., e/g-mediated and 4-mediated interfaces. In this case, an oligomeric arrangement of the ATP synthase involving these two interfaces is conceivable (*10*, *11*). Such an arrangement has been visualized by BN-PAGE in digitonin extracts of yeast and mammalian mitochondria (*10*, *12*). Interestingly ATP synthase oligomerization has been proposed to play a role in the morphogenesis of the mitochondrial inner membrane cristae (*13*, *14*). Furthermore, correct supramolecular organization of the ATP synthase complexes is crucial for the genesis and/or the maintenance of the mitochondrial morphology (*10*, *11*, *15*).

In this study, we focus on the possible role of subunits h and i in the ATP synthase dimerization/oligomerization process. Subunit h is a 10.4 kDa water-soluble ATP synthase subunit encoded by the nuclear gene *ATP14* (*16*), exhibiting a stoichiometry of 1 subunit h per ATP synthase complex (*17*). Inactivation of the *ATP14* gene leads to a lack of oxidative phosphorylation and high instability of the mitochondrial DNA, thus suggesting that subunit h plays an essential role in assembly and/or stability of the F_1F_o ATP synthase. Complementation experiments in *S. cerevisiae* showed that subunit h is the functional homologue of the mammalian F6 subunit (*18*). Subunit h has been localized in the vicinity of subunits α, 4, d, and f and was therefore assigned to the peripheral stalk of the enzyme (*17*). From topological analyses, a structural model for the yeast subunit h was proposed (*17*) and further confirmed by the NMR structure determination of the bovine homologue F6 (*19*). This subunit is composed of two α-helix domains separated by a central loop, the C-terminal domain being unstructured and flexible. Biotinylation of subunit h and electron microscopy analysis of ATP synthase−avidin complexes suggested that its C-terminus is located close to the point at which the peripheral stalk emerges from the membrane (*20*). Recently, a model was proposed for the bovine ATP synthase peripheral stalk in which subunit F6 (subunit h in yeast) is fully extended along subunit 4 (*21*).

Although its arrangement and its exact positioning in the peripheral stalk have not clearly been defined (*17*, *19*), this subunit occupies a central position in the peripheral stalk that may confer an important role in the physical coupling between F_1 and F_o and in peripheral stalk structure stability. In a previous work, we have suggested that subunit h could dimerize through disulfide bridge formation and may be located at the dimerization interface between two F_1F_o ATP synthases (*17*).

Subunit i is encoded by the nuclear gene *ATP18* and is an integral membrane subunit of the ATP synthase (*22*). Although it displays a proton leak at the ATP synthase level, the null mutant Δ*atp18* is able to grow on nonfermentable media, thus indicating that the protein is not essential (*22*). Study of the subunit i environment showed a proximity between the unique membrane spanning segment of subunit i and the first transmembrane α-helix of subunit 6 (*23*). This study also revealed proximities between subunit i and subunits d, and f, which are components of the peripheral stalk, and the supernumerary subunit g (*23*). Finally, subunit i, whose stoichiometry is 1 per complex, was shown to be able to form a homodimer (i + i) by chemical cross-linking. This dimerization occurs only within the ATP synthase dimer (*11*, *24*).

In this study, we confirm the ability of subunit h to form a homodimer through disulfide bridge formation within the ATP synthase dimer, indicating a close proximity of the peripheral stalks in the dimer. Moreover, we found that homodimerization of both subunit h and subunit i was independent of the presence of subunits e and g, providing direct evidence of ATP synthase's ability to dimerize in the absence of these subunits.

EXPERIMENTAL PROCEDURES

Materials. Triton X-100 and digitonin were from Sigma. All other chemicals were of reagent grade quality.

Construction of Mutant Strains. The *S. cerevisiae* strain D273-10B/A/H/U (*MAT*α, *met6*, *his3*, *ura3*) was the control wild-type strain (*25*). For clarity, the yeast mutants were named as (mutated subunit)-(wild-type residue)(residue number)(mutant residue), where the residues were named by their single letter code. The label -H6 was added at the end of the name when a C-terminal polyhistidine tag was added. The strains containing modified versions of subunit h were obtained after complementation of the disrupted yeast strain JVY1-2 (*MAT*α, *met6*, *his3*, *ura3*, *atp14::URA3*) by the monocopy vector pRS313 containing each different mutated *atp14* gene. Lysine to cysteine residue replacement was done by overlap extension using PCR (*26*) with the wild-type *ATP14* gene as template. The mutant h-K36C-H6 was obtained by addition of five histidine residues to the C-terminal histidine present in subunit h using the *atp14*-K36C mutant gene as template. The strains Δ*tim11* h-K36C and Δ*tim11* i-K51C were constructed from h-K36C (*17*) and i-K51C strains, respectively (*23*). The *TIM11* gene was disrupted by the *Kan*^{r} gene using a PCR-based method (*27*). Bacteria and yeast were respectively transformed by electroporation and lithium chloride methods (*28*).

Biochemical Procedures. Cells were grown aerobically at 28 °C in a complete liquid medium containing 2% lactate as carbon source and harvested in logarithmic growth phase (8×10^7 cells/mL). Mitochondria were prepared as described previously (*29*) and suspended in the isolation buffer (0.6 M mannitol, 2 mM EGTA, 10 mM Tris−maleate, pH 6.8). Protein concentration was determined according to the Lowry method (*30*) in the presence of 5% (w/v) SDS with bovine serum albumin as standard protein.

Disulfide Bridge Formation Experiments. Mitochondria were washed twice and resuspended in 0.6 M mannitol and 50 mM HEPES, pH 7.4, at a protein concentration of 5 mg/mL. This suspension was incubated in the same buffer in the absence or in the presence of 1 or 2 mM $CuCl_2$. Reactions were stopped after 30 min incubation at 4 °C by addition of 5 mM EDTA and 5 mM NEM. Measurement of the ATPase activity in mitochondria from the strains used in this work, treated or not with $CuCl_2$, had shown that such a treatment had almost no effect since only a slight decrease in oligomycin sensitivity of about 5% was observed in treated

mitochondria as compared to untreated mitochondria (data not shown).

Protein Extracts. The mitochondrial Triton X-100 extracts were prepared as in Paumard et al. (*23*). The digitonin extracts were prepared at a digitonin to protein ratio of 0.75 g/g to 2 g/g in 30 mM HEPES pH 7.4, 150 mM potassium acetate, 2 mM 6-aminohexanoic acid, 10% (m/v) glycerol, and proteases inhibitors with or without 1 mM EDTA.

Electrophoretic and Western Blot Analyses. SDS−PAGE was performed on Tris−tricine 15% polyacrylamide slab gels according to Schägger and von Jagow (*31*). Silver nitrate staining was performed as described (*32*, *33*). BN-PAGE experiments were carried out as described by Schägger (*34*, *35*). The mitochondrial digitonin extracts were centrifugated at 4 °C for 15 min at 40000*g*. The supernatant was immediately loaded onto the top of a 3−13% polyacrylamide slab gel. After electrophoresis the gel was incubated in a solution of 5 mM ATP, 5 mM $MgCl_2$, 0.05% (w/v) lead acetate, and 50 mM glycine−NaOH, pH 8.6, to detect ATPase activity (*36*). The bands were cut, incubated in 2% (w/v) SDS for 1 h, and analyzed in a second dimension by SDS−PAGE (*31*). Western blot analyses have been described previously (*16*). Evaluation of the apparent molecular mass of cross-linked products was done using molecular masses of prestained protein ladder from Invitrogen Corp. Proteins were electrotransferred onto nitrocellulose membranes (Membrane Protean BA83, Schleicher and Shuell). Primary antibodies were polyclonal rabbit antibodies. Secondary antibodies were peroxidase-conjugated goat anti-rabbit antibodies (Jackson Immuno Research Laboratories, Inc.). Western blot analyses were done using the Enhanced Chemio Luminescence method (Amersham Pharmacia Biotech).

Anti-Subunit h Antibody Purification. Anti-h serum was prepared by rabbit immunization with subunit h purified by HPLC as described previously (*16*). Polyclonal anti-subunit h antibodies were purified by affinity FPLC as described previously (*17*).

Ni-NTA Purification of Cross-Linked Products. Purification of H6-tagged proteins was done in denaturing conditions [0.6 M mannitol, 0.1% (w/v) SDS, 50 mM HEPES−NaOH, pH 7.0] as in Valiyaveetil and Fillingame (*37*).

Nanospray LC−MS Analyses. Protein samples were analyzed by on-line capillary chromatography (LC Packings, Amsterdam, The Netherlands) coupled to a nanospray LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Proteins were separated on a 75 μm inner diameter × 15 cm C18 PepMap column (LC Packings, Amsterdam, The Netherlands). The flow rate was set to 200 nL/min. Proteins were eluted in 30 min using a 5−50% linear gradient of solvent B (solvent A was 0.1% formic acid in 5% acetonitrile, and solvent B was 0.1% formic acid in 80% acetonitrile). The mass spectrometer was operated in positive ion mode at a 2 kV needle voltage and a 46 V capillary voltage. Full scan MS were collected over the range *m*/*z* 50−2000.

RESULTS

Subunit h Dimer Formation by Oxidation. In a previous work (*17*), we showed that when several subunit h cysteine mutant mitochondria (h-K12C, h-K29C, h-K36C, h-K51C, and h-K65C) were incubated with $CuCl_2$, a 24 kDa band revealed by the anti-h antibodies appeared on Western blots.

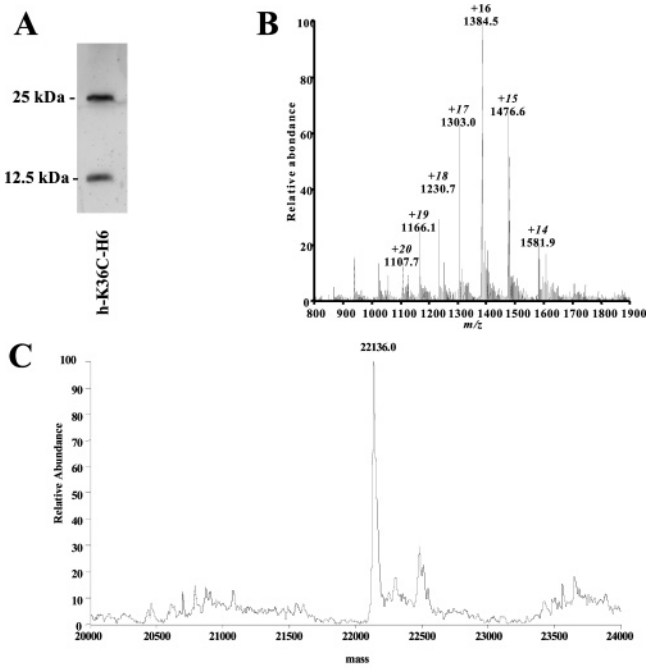

FIGURE 1: Homodimer formation of subunit h confirmed by mass spectrometry analysis. Mitochondria from the subunit h-K36C-His_6 mutant were incubated with $CuCl_2$ to promote disulfide bridge formation. The His_6-tagged protein and adduct were purified by Ni-NTA affinity chromatography after membrane solubilization in 1% (w/v) SDS as described in Experimental Procedures. The purified fraction was analyzed by silver-stained SDS−PAGE (A). The same sample was also separated by capillary liquid chromatography coupled to mass spectrometry. The mass spectrum of the 25 kDa product was established (B), making it possible to determine its exact mass (C).

We then proposed that the 24 kDa cross-linked product was a dimer of subunits h linked by a disulfide bridge. To confirm this hypothesis, the subunit h-K36C-H6 mutant cross-linked product was purified by Ni-NTA affinity chromatography, separated by SDS−PAGE, and analyzed by mass spectrometry after "in gel" trypsin digestion. This analysis showed that all but two of the peptides present in the sample derived from subunit h (17). As two unidentified peptides were also detected, it was not possible, with this method, to absolutely confirm the existence of the subunit h dimer. Therefore, the exact mass of the Ni-NTA purified proteins was measured by nanoESI mass spectrometry coupled to microHPLC separation. After $CuCl_2$ oxidation of h-K36C-H6 mitochondria and Ni-NTA purification, only two bands of 12.5 and 25 kDa apparent mass were revealed on Western blots by the anti-subunit h antibodies (not shown) or by silver staining of the gel (Figure 1A). Mass spectrometry analysis showed that the major proteic entity present in solution had a mass of 22136 ± 2 Da (Figure 1C). This mass corresponded exactly to the mass of two subunits h-K36C-H6 linked by a disulfide bridge. A second component was detected with a mass of 11073 ± 5 Da (data not shown) and corresponded to the subunit h-K36C-H6 monomer (expected mass 11068 Da). This result confirmed the formation by oxidation of subunit h dimers in mitochondria. As subunit h stoichiometry is 1 per ATP synthase complex (*17*), the dimerization of this subunit should involve two monomers of ATP synthase.

Spannagel et al. (*5*) and Paumard et al. (*10*) have shown, using subunit 4 cysteine mutants, that subunit 4 dimer

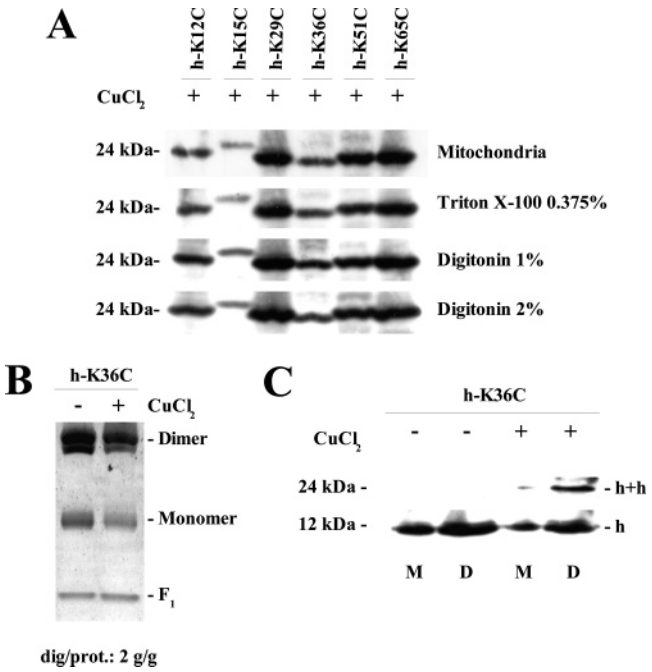

FIGURE 2: Subunit h dimerization and ATP synthase dimerization are linked. (A) Mitochondria from yeast mutants h-K12C, h-K15C, h-K29C, h-K36C, h-K51C, and h-K65C were incubated with 1 mM $CuCl_2$ for 30 min at 4 °C. Triton X-100 and digitonin extractions were performed as described in Experimental Procedures. 50 μg of mitochondrial protein extracts was separated by SDS−PAGE and analyzed by Western blot. Blots were probed with anti-h purified polyclonal antibody (dilution 1:500). For clarity, only the 24 kDa band is displayed. In the absence of $CuCl_2$, this band is absent in mitochondria and protein extracts of all the mutants. When mitochondria from the wild-type strain were used, the 24 kDa bands were not detectable either in the absence or in the presence of $CuCl_2$. (B) Mitochondria from the h-K36C mutant strain, incubated or not with $CuCl_2$, were solubilized at a digitonin to protein ratio of 2 g/g. The mitochondrial complexes were separated by BN-PAGE, and gels were incubated with ATP−Mg^{2+} and Pb^{2+} to reveal the ATPase activity. The figure is the negative of the original gels. (C) The bands corresponding to the monomeric and dimeric forms of ATP synthase from the gel displayed in (B) were cut out, and the proteins they contained were separated by SDS−PAGE, analyzed by Western blot, and revealed by purified anti-h antibodies (dilution 1:500). M = monomer; D = dimer.

homodimers could be formed in the mitochondrial membrane, indicating that subunit 4 may represent one dimerization interface of the ATP synthase and may participate in the formation of oligomers (*10*). However, this hypothesis could not be confirmed since subunit 4 dimers are resistant to extraction by detergents such as Triton X-100 or digitonin that are otherwise able to solubilize ATP synthase oligomers and dimers (*10*).

For each subunit h cysteine mutant, the sensitivity of the subunit h dimer band (24 kDa) to the detergent extraction was studied by Western blot. After $CuCl_2$ oxidation performed on isolated mitochondria, the mitochondrial proteins were extracted with either Triton X-100 or digitonin, separated by SDS−PAGE, and transferred onto nitrocellulose membrane. When this experiment was performed on wild-type mitochondria or in the absence of $CuCl_2$, the 24 kDa band was absent (not shown). After $CuCl_2$ oxidation and extraction with either digitonin (1 g/g protein and 2 g/g protein) or Triton X-100 (0.375 g/g protein), the 24 kDa band was detected on Western blot, with the anti-h antibodies, for all of the cysteine mutants (Figure 2A). These results showed that an h + h homodimer could be formed from each introduced cysteine residue and that the h + h dimers could be solubilized by mild detergents. It was then possible to verify that subunit h dimerization is linked to ATP synthase dimerization. After $CuCl_2$ oxidation of subunit h-K36C mutant mitochondria, membrane complexes were extracted by digitonin and immediately separated by Tris−tricine BN-PAGE. ATPase activity was revealed in the gel as described in Experimental Procedures. At a digitonin to protein ratio of 2 g/g, wild-type extracts contained essentially the dimeric form of ATP synthase and to a lesser extent the monomeric form and the F_1 sector (*10*). Similar results were obtained with the h-K36C mutant extracted in the same condition (Figure 2B). The bands corresponding to monomeric or dimeric forms of ATP synthase were cut out and submitted to a second dimension SDS−PAGE separation, and proteins were transferred onto nitrocellulose. As shown on Figure 2C, the h + h homodimer band was mainly present in the dimeric form of the ATP synthase when the complexes were extracted with 2 g of digitonin/g of protein. When lower concentrations of digitonin were used (i.e., 1 g/g), the subunit h dimer was visible only in the ATP synthase dimer (not shown). The low amount of subunit h dimer present in the ATP synthase monomer (Figure 2C) might be due to the high digitonin concentration that could destabilize the ATP synthase dimer. In the absence of $CuCl_2$, no subunit h dimers were detected (Figure 2C). These results demonstrate that h + h homodimer formation occurs in the ATP synthase dimer.

Subunit h Homodimerization Is Independent of the Presence of Subunits e and g. It has been shown that subunits e and g are highly involved in the ATP synthase dimerization process. Arnold et al. showed these two subunits to be essential for observing the dimeric form of the ATP synthase after detergent extraction and BN-PAGE separation (*7*). To determine whether subunit h dimerization was dependent or not on the presence of subunits e and g, the Δ*tim11* h-K36C strain was constructed. Note that, in the absence of subunit e, subunit g cannot be assembled in the complex and is degraded (*10*). As expected, subunits e and g were not detected in Δ*tim11* h-K36C (Figure 3A and not shown). After $CuCl_2$ oxidation of mitochondria isolated from this strain, the 24 kDa band corresponding to the h + h homodimer was still present in the Δ*tim11* h-K36C mitochondria despite the absence of subunits e and g (Figure 3A). Note that the proportion of the subunit h involved in the dimer is higher in the absence of subunits e and g than in their presence.

Subunit h Homodimerization Stabilizes ATP Synthase Dimers in the Absence of Subunits e and g. To determine whether the h + h homodimer was associated to ATP synthase complexes, mitochondria from the Δ*tim11* h-K36C strain were incubated or not with $CuCl_2$, and the complexes from the inner membrane were extracted by digitonin (1 g/g of protein or 2 g/g of protein) and separated by BN-PAGE. The presence of the ATP synthase complexes was revealed by their ATPase activity as described in Experimental Procedures. In the absence of $CuCl_2$, no ATP synthase dimers were detected in Δ*tim11* h-K36C mitochondria (Figure 3B). In some experiments, a very faint band corresponding to the ATP synthase dimer could be observed, e.g., Δ*tim11* mitochondria (not shown). This faint band corresponds to a very low amount of ATP synthase dimer since it was not stained by Coomassie Brilliant blue. Very low levels of ATP

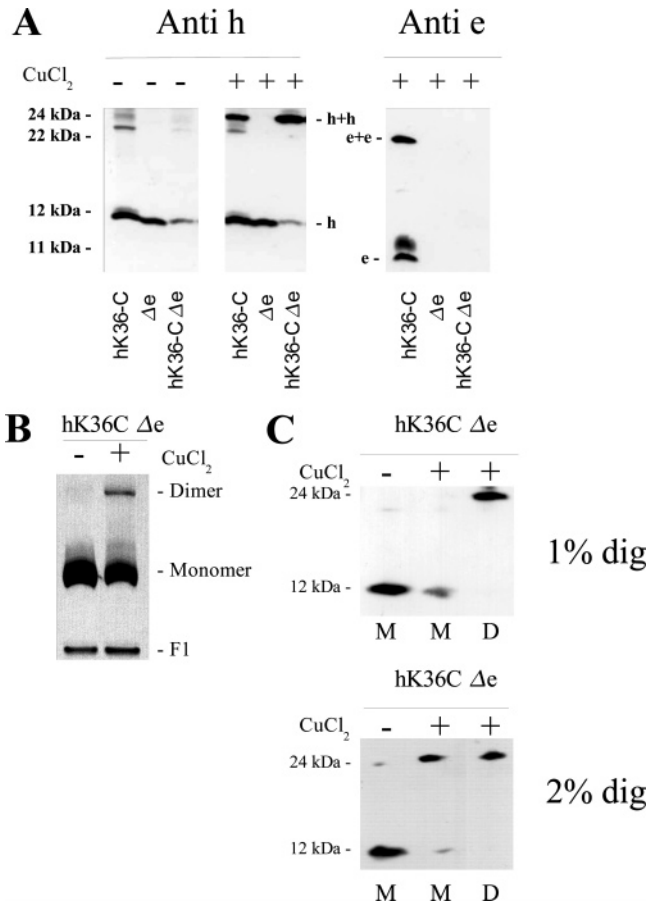

FIGURE 3: Dimerization of subunit h occurs in the absence of subunits e and g. (A) Mitochondria from the h-K36C, Δ*tim11*, and Δ*tim11* h-K36C mutant strains were incubated or not with 1 mM $CuCl_2$ as described in Experimental Procedures. Samples (50 μg of mitochondrial proteins) were separated by SDS−PAGE, analyzed by Western blot, and revealed by purified anti-h antibodies (dilution 1:500) and anti-e polyclonal serum (dilution 1:10000). (B) Mitochondria from the Δ*tim11* h-K36C mutant strain were incubated or not with $CuCl_2$ and solubilized at a digitonin to protein ratio of 2 g/g. After centrifugation, the mitochondrial complexes were separated by BN-PAGE, and gels were incubated with ATP−Mg^{2+} and Pb^{2+} to reveal the ATPase activity. The figure is the negative of the original gels. (C) The bands corresponding to the monomeric and dimeric forms of ATP synthase from BN-PAGE obtained from extracts done at a digitonin to protein ratio of 1 g/g or 2 g/g were cut out, and the proteins they contained were separated by Tris-Tricine SDS−PAGE, analyzed by Western blot, and revealed by purified anti-h antibodies (dilution 1:500). M = monomer; D = dimer.

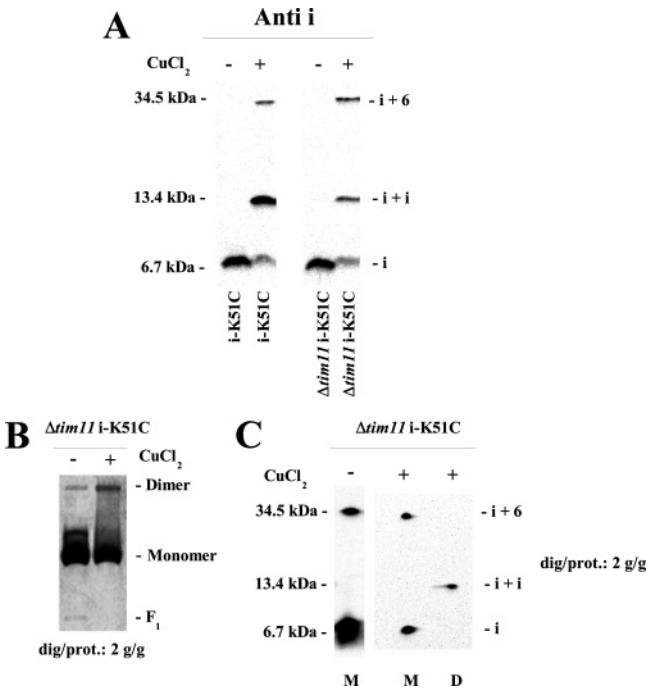

FIGURE 4: Dimerization of subunit i occurs in the absence of subunits e and g. (A) Mitochondria from the i-K51C and Δ*tim11* i-K51C mutant strains were incubated or not with 1 mM $CuCl_2$ as described in Experimental Procedures. Samples (50 μg of mitochondrial proteins) were separated by SDS−PAGE, analyzed by Western blot, and revealed by anti-i antibodies (dilution 1:10000). (B) Mitochondria from the Δ*tim11* i-K51C mutant strain were incubated or not with $CuCl_2$ and solubilized at a digitonin to protein ratio of 2 g/g. After centrifugation, the mitochondrial complexes were separated by BN-PAGE, and gels were incubated with ATP−Mg^{2+} and Pb^{2+} to reveal the ATPase activity. The figure is the negative of the original gels. (C) The bands corresponding to the monomeric and dimeric forms of the ATP synthase from the BN-PAGE displayed in (B) were excised, separated in a second dimension by SDS−PAGE, analyzed by Western blot, and revealed by anti-i antibodies (1:10000). M = monomer; D = dimer.

synthase dimer have been observed by BN-PAGE in strains lacking subunit g (*8*, *11*). When $CuCl_2$ oxidation was performed on these mitochondria before digitonin extraction, a band corresponding to a significant amount of ATP synthase dimer was detected either by ATP activity staining (Figure 3B) or by Coomassie blue staining (not shown). The bands corresponding to the ATP synthase monomer and dimer were excised, and the ATP synthase subunits were separated in a second dimension by SDS−PAGE, transferred onto nitrocellulose membrane, and probed with anti-subunit h antibodies. When the extraction was performed at a low detergent to protein ratio (1 g/g of protein), the h + h homodimer was detectable only in the ATP synthase dimer and there was no subunit h monomer in the ATP synthase monomer band (Figure 3C). Thus, the ATP synthase dimer observed by BN-PAGE was the sole subunit h-mediated ATP synthase dimer. At higher digitonin to protein ratio (2 g/g of protein), the h + h homodimer was present in both the monomer and the dimer of ATP synthase (Figure 3C). Under these conditions, it is possible that the ATP synthase dimer where two subunits h are linked by a disulfide bridge is unstable and dissociated into monomers, one of them containing the h + h cross-linked homodimer. In any case, for the first time, ATP synthase subunit e/g independent dimers could be stabilized and clearly visualized by BN-PAGE.

ATP Synthase Dimer Stabilization via Subunit i Homodimerization in the Absence of Subunits e and g. In 2002, Paumard et al. showed the presence of subunit i dimers in the mitochondrial inner membrane and provided evidence of the link between this dimerization and the F_1F_o ATP synthase dimerization (*10*). To determine whether the dimerization of subunit i in mitochondria depends on the presence of subunits e and g, the Δ*tim11* i-K51C strain was constructed. Mitochondria isolated from either i-K51C or Δ*tim11* i-K51C strains were submitted or not to copper oxidation. Western blot analyses were performed and confirmed that subunits e and g were absent in Δ*tim11* i-K51C mitochondria (not shown). As already described (*24*) and shown in Figure 4A, after $CuCl_2$ incubation of i-K51C mitochondria, 12 and 29 kDa oxidation products were obtained. They corresponded

to an i + i homodimer and to an i + 6 heterodimer (via Cys23 of subunit 6), respectively. Upon $CuCl_2$ oxidation of the Δ*tim11* i-K51C mitochondria, the i + i homodimer and i + 6 heterodimer were still observed (Figure 4A). Nevertheless, in comparison to the wild-type context, the absence of subunits e and g produced a weaker signal for i + i, while the i + 6 signal was enhanced. The relationship between the i + i dimer observed in mitochondria lacking subunits e and g and the dimerization of F_1F_o ATP synthase was analyzed. BN-PAGE was performed on digitonin extracts from Δ*tim11* i-K51C mitochondria, incubated or not with $CuCl_2$. When digitonin solubilization was performed on nonoxidized Δ*tim11* i-K51C mitochondria, a very faint band of ATP synthase dimer was detected and revealed only by its ATPase activity (Figure 4B). This was also the case for nonoxidized Δ*tim11* h-K36C (Figure 3B) and for Δ*tim11* mitochondria (*10*). When the oxidation step preceded digitonin extraction, a significant amount of ATP synthase dimer was revealed, not only by its ATPase activity (Figure 4C) but also by Coomassie blue staining (not shown). Slices of gel containing monomeric and dimeric F_1F_o ATP synthase were submitted to SDS−PAGE, and Western blots with anti-i antibodies were performed (Figure 4C). Whereas monomeric subunit i and i + 6 heterodimers were found in the monomer of ATP synthase, the i + i homodimer was exclusively found in the dimeric form of the enzyme. This specificity proved that, in mitochondria devoid of the subunits e and g, the dimerization of subunit i reflects a dimeric organization of the ATP synthase.

DISCUSSION

Proximity of the Peripheral Stalks in the ATP Synthase Dimer. In a previous work, using lysine to cysteine replacement and either chemical cross-linking or disulfide bridge formation, we suggested that the subunit h of the ATP synthase could form homodimers (*17*). Direct measurement of the mass of the subunit h cross-linked product confirmed this hypothesis. The formation of the h + h homodimer could be obtained from several mutants in which a cysteine residue was introduced in different positions in the primary structure. As this subunit stoichiometry is 1 per F_1F_o complex (*17*), these results indicate that subunit h is located at the periphery of the enzyme and near a second subunit h. This is consistent with the proposed topology of subunit h within the peripheral stalk (*17*).

Thus subunit h belongs to a class of ATP synthase subunits that are able to form homodimers by chemical cross-linking, including subunit 4 (*5*), subunit i (refs *11* and *24* and this work), subunit e (*38*, *39*), and subunit g (*40*, *41*). The last two subunits have been shown to be involved in the dimerization process of the F_1F_o ATP synthase (*7*, *10*). As subunits 4, h, and i have a stoichiometry of 1 per ATP synthase complex (*5*, *17*, *23*), their ability to form homodimers strongly suggests the presence of ATP synthase dimers within the inner mitochondrial membrane and could indicate a close proximity of these subunits in the dimer. Unfortunately, the involvement of subunit 4 in the enzyme dimerization could only be studied in intact mitochondria preparations (*10*) or in vivo (*9*). So far, it has not been possible to perform further biochemical studies since the 4-D54C 4 + 4 homodimer cannot be solubilized by mild detergents, such as digitonin, which is able to extract and maintain in solution the monomeric, dimeric, and oligomeric forms of the entire complex (*10*). The constraints imposed on two F_o sectors chemically linked by two subunits 4 most likely do not allow the solubilization of such a dimer. On the other hand, Gavin et al. (*9*), using coexpression of GFP- and BFP-tagged subunit 4 and FRET measurements, have demonstrated the proximity between two subunits 4 in the mitochondrial membrane. However, due to the low resolution of FRET experiments (100−200 Å), these results could not indicate a direct interaction between two subunits 4. In contrast to what was observed with subunit 4, homodimers of subunits h and i could be extracted by digitonin. Moreover, they were found associated to the dimeric form of ATP synthase (Figures 2 and 4 and ref *24*), providing clear evidence that the subunits h and i are located near or participate in the dimerization interface. Since it is now clearly established that subunit h belongs to the peripheral stalk (*17*, *20*), this result is the first direct, biochemical evidence for a close proximity between two peripheral stalks within the dimer and thus suggests a possible role of this stalk in the dimerization process.

ATP Synthase Dimers in the Absence of Subunits e and g. It has been clearly established that subunits e and g are directly involved in the dimeric organization of the ATP synthase in the inner mitochondrial membrane (*7*, *10*). These two subunits were shown to be associated to the dimeric and oligomeric forms of the ATP synthase. The absence of either subunit g alone or both subunits e and g (*7*, *10*, *11*) or the introduction of deletions or mutations especially in the GXXXG motif present in their transmembrane domain (*38*−*41*) results in the destabilization of the dimerization interface since, in all of these conditions, almost all of the ATP synthases were found monomeric after digitonin extraction and BN-PAGE. From these observations, subunits e and g were proposed as dimerization subunits.

The results presented in this paper showed that homodimerization of subunit h and subunit i could be obtained in a Δ*tim11* context. Thus ATP synthase dimers are still present in the inner mitochondrial membrane despite the absence of subunits e and g. This confirms the results obtained by Gavin et al., who observed FRET between subunit 4−GFP and subunit 4−BFP fusions in cells lacking subunit e (*9*). Moreover, chemical cross-linking between subunits h or subunits i belonging to two monomers allowed extraction by digitonin and visualization by BN-PAGE of ATP synthase dimers devoid of subunits e and g. These observations suggest that subunits e and g are involved in the stabilization of the ATP synthase dimers and may not be necessary for their formation. In the absence of these two subunits, the dimerization interface is most likely less resistant to detergent extraction. The very thin band of dimeric form detected by ATPase activity in BN-PAGE obtained from Δ*tim11* digitonin extracts may correspond to a few dimers that were not dissociated by the detergent. Recently, Rexroth et al. (*42*) have shown that a dimeric form of the chloroplastic ATP synthase from *Chlamydomonas reinhardtii* could be visualized in BN-PAGE. These authors suggested that dimerization may be a general feature of all H^+-ATP synthase complexes and may involve the peripheral stalk. It should be noted that no homologues of subunits e and g have been found in the chloroplast although the presence of unidentified counterparts cannot be excluded.

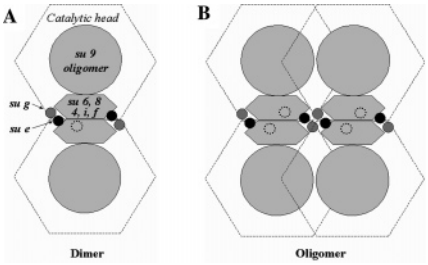

FIGURE 5: A model for the supramolecular organization of the ATP synthase. (A) organization of the dimerization interface in the membrane seen from the intermembrane space perpendicular to the main axis of the ATP synthase. The area in gray represents the cross section of the subunit 9 oligomer and of the F_o domain. The F_1 sector is represented as a dashed line. The dimerization interface involves a large part of the F_o sector and may also extend to components localized in the matrix, such as subunit h (dot line). Subunits e and g are localized at the periphery of the dimerization interface and stabilize it. In the dimer, subunits e and g are in close contact on each side of the dimer. (B) Oligomerization of ATP synthase may occur by interaction between the e + g interfaces.

Noticeably, the amounts of dimer stabilized by h + h or i + i homodimers detected in BN-PAGE (Figures 3B and 4B) are lower than one could expect from the proportion of subunit h or subunit i involved in the cross-linking (Figures 3A and 4A). This might be due to the fact that dimers linked by a disulfide brigde are partially resistant to digitonin solubilization or less stable in detergent solution than native dimers.

An Improved Model for Supramolecular Organization of the ATP Synthase in the Mitochondrial Inner Membrane. On the basis of the early work of Allen (*13*, *14*) and the observation that the absence of subunits e and g destabilized the supramolecular organization of the ATP synthase and dramatically altered the mitochondrial morphology, Paumard et al. (*10*) have proposed a model for the dimerization/oligomerization of the ATP synthase. This model defines two distinct dimerization interfaces, one mediated by subunits e and g and the other by subunit 4. Homodimerization and heterodimerization of subunits e and g were recently shown by cross-linking using endogenous and/or introduced cysteine residues (*38*−*41*). The e + g heterodimer is specific to the dimeric form of the ATP synthase, and the e + e and g + g homodimers are specific to the oligomeric form (probably tetrameric) (*38*, *40*). Moreover, cross-linking of e + e or g + g homodimers before detergent extraction stabilizes oligomeric forms of the ATP synthase that remained present at high digitonin to protein ratio (*38*, *40*). These data suggest that subunits e and g may also play a role in the formation of oligomeric forms of the ATP synthase in the mitochondrial membrane.

Figure 5 represents an improved model that takes into account the results described in this paper and those from the studies on subunit e and g dimerization (*38*, *40*). Data presented in this paper suggest that subunits e and g are not required for the formation of dimers but rather are necessary for their stabilization in the membrane and in detergent extracts. Thus, these two subunits may be located at the periphery of the dimerization interface. Two ATP synthase monomers may be in interaction via their peripheral stalk through a large dimerization interface.

As they can form homodimers in the membrane, subunit 4 and subunit i may be involved in the dimerization interface. The subunit h, which is located in the matrix, may also be involved in or be located in close proximity to the dimerization interface. In the membrane, subunits e and g interact with each other and with other subunits to stabilize the dimer (Figure 5A). In the absence of subunit e and/or subunit g, the dimerization interface may still exist but is more sensitive to detergents. Oligomerization may result from interactions between these two subunits located at the periphery of the dimers (Figure 5B). Such a model defines two interfaces: the first one for the dimerization and the second one for the oligomerization. In contrast to the previous model (*9*, *10*), these two interfaces are not independent since subunits e and g stabilize the first one and are directly involved in the second one. Note that adjacent dimers must be tilted one from the other in order to avoid physical contact between the F_1 sectors. An angle of at least 20° may be sufficient to avoid such a contact. Such an organization will result in a curvature of the membrane that may be involved in the mitochondrial inner membrane morphology.

Recent studies using electron microscopy and single particle analysis were performed on ATP synthase dimers isolated from beef heart mitochondria (*43*) and from mitochondria of the alga *Polytomella* (*44*). Both studies showed that, in solubilized dimers, two monomers are linked at least by the F_o domain and are clearly separated by an angle of 40° for the beef ATP synthase and 70° for *Polytomella* ATP synthase. In the beef enzyme, the two F_1 domains are in close proximity and are connected by protein bridges that might involve the peripheral stalk at the matrix side and F_o subunits at the intermembrane space side (*43*). In the ATP synthase dimer from *Polytomella*, the peripheral stalk is clearly visible between the two monomers (*44*). Our results are consistent with these observations since disulfide bridges could be obtained between two F_o domain subunits i, belonging to two monomers (Figure 4). Moreover, our results suggest that the dimerization interface contains not only the F_o domain but may extend to the peripheral stalk since subunit h is able to form homodimers within the membrane (Figures 2 and 3). The dimeric form of the yeast enzyme may be organized as the beef enzyme, in which the proximity between peripheral stalks and the angle between the monomers (*43*) are consistent with our data and our model (Figure 5).

In conclusion, results presented in this study provide direct evidence of a close proximity between two peripheral stalks in the ATP synthase dimer. They also demonstrate that ATP synthase dimers are present in the mitochondrial inner membrane even in the absence of subunits e and g and suggest that subunits from the peripheral stalk may participate in the dimerization interface. However, since the formation of a disulfide bridge between two subunits indicates that they are in close proximity but is not a proof of a physical specific interaction, the direct participation of subunits h and i in the dimerization process, which our results might suggest, remains to be established. Work is in progress in order to better define the dimerization interface(s) at the molecular level and the role of the different subunits in the dimerization process.

ACKNOWLEDGMENT

The authors are grateful to Dr. Marie-France Giraud for critical reading of the manuscript, to Dr. Diego Bustos and

Dr. Esther Marza for stimulating discussions, and to Joanne Pageze and Dr. Christine Schwimmer for reading the English version of the manuscript. The authors acknowledge Dr. Stéphane Claverol and Prof. Marc Bonneu from the Plateforme de Génomique Fonctionnelle at the Université Victor Segalen Bordeaux 2 for the LC-MS analysis.

REFERENCES

1. Velours, J., and Arselin, G. (2000) The *Saccharomyces cerevisiae* ATP synthase, *J. Bioenerg. Biomembr. 32*, 383−390.
2. Boyer, P. D. (1993) The binding change mechanism for ATP synthase—some probabilities and possibilities, *Biochim. Biophys. Acta 1140*, 215−250.
3. Hausrath, A. C., Capaldi, R. A., and Matthews, B. W. (2001) The conformation of the epsilon- and gamma-subunits within the *Escherichia coli* F(1) ATPase, *J. Biol. Chem. 276*, 47227−47232.
4. Fillingame, R. H., and Dmitriev, O. Y. (2002) Structural model of the transmembrane Fo rotary sector of H^+-transporting ATP synthase derived by solution NMR and intersubunit cross-linking in situ, *Biochim. Biophys. Acta 1565*, 232−245.
5. Spannagel, C., Vaillier, J., Arselin, G., Graves, P. V., Grandier-Vazeille, X., and Velours, J. (1998) Evidence of a subunit 4 (subunit b) dimer in favor of the proximity of ATP synthase complexes in yeast inner mitochondrial membrane, *Biochim. Biophys. Acta 1414*, 260−264.
6. Bateson, M., Devenish, R. J., Nagley, P., and Prescott, M. (1999) Single copies of subunits d, oligomycin-sensitivity conferring protein, and b are present in the *Saccharomyces cerevisiae* mitochondrial ATP synthase, *J. Biol. Chem. 274*, 7462−7466.
7. Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., and Schägger, H. (1998) Yeast mitochondrial F_1F_o-ATP synthase exists as a dimer: identification of three dimer-specific subunits, *EMBO J. 17*, 7170−7178.
8. Schägger, H. (2001) Respiratory chain supercomplexes, *IUBMB Life 52*, 119−128.
9. Gavin, P. D., Prescott, M., and Devenish, R. J. (2005) F_1F_o-ATP synthase complex interactions in vivo can occur in the absence of the dimer specific subunit e, *J. Bioenerg. Biomembr. 37*, 55−66.
10. Paumard, P., Vaillier, J., Coulary, B., Schaeffer, J., Soubannier, V., Mueller, D. M., Brèthes, D., di Rago, J. P., and Velours, J. (2002) The ATP synthase is involved in generating mitochondrial cristae morphology, *EMBO J. 21*, 221−230.
11. Giraud, M. F., Paumard, P., Soubannier, V., Vaillier, J., Arselin, G., Salin, B., Schaeffer, J., Brèthes, D., di Rago, J. P., and Velours, J. (2002) Is there a relationship between the supramolecular organization of the mitochondrial ATP synthase and the formation of cristae?, *Biochim. Biophys. Acta 1555*, 174−180.
12. Krause, F., Reifschneider, N. H., Goto, S., and Dencher, N. A. (2005) Active oligomeric ATP synthases in mammalian mitochondria, *Biochem. Biophys. Res. Commun. 329*, 583−590.
13. Allen, R. D., Schroeder, C. C., and Fok, A. K. (1989) An investigation of mitochondrial inner membranes by rapid-freeze deep-etch techniques, *J. Cell. Biol. 108*, 2233−2240.
14. Allen, R. D. (1995) Membrane tubulation and protons pumps, *Protoplasma 189*, 1−8.
15. Gavin, P. D., Prescott, M., Luff, S. E., and Devenish, R. J. (2004) Cross-linking ATP synthase complexes in vivo eliminates mitochondrial cristae, *J. Cell. Sci. 117*, 2333−2343.
16. Arselin, G., Vaillier, J., Graves, P. V., and Velours, J. (1996) ATP synthase of yeast mitochondria. Isolation of the subunit h and disruption of the ATP14 gene, *J. Biol. Chem. 271*, 20284−20290.
17. Fronzes, R., Chaignepain, S., Bathany, K., Giraud, M. F., Arselin, G., Schmitter, J. M., Dautant, A., Velours, J., and Brèthes, D. (2003) Topological and functional study of subunit h of the F_1F_o ATP synthase complex in yeast *Saccharomyces cerevisiae*, *Biochemistry 42*, 12038−12049.
18. Velours, J., Vaillier, J., Paumard, P., Soubannier, V., Lai-Zhang, J., and Mueller, D. M. (2001) Bovine coupling factor 6, with just 14.5% shared identity, replaces subunit h in the yeast ATP synthase, *J. Biol. Chem. 276*, 8602−8607.
19. Carbajo, R. J., Silvester, J. A., Runswick, M. J., Walker, J. E., and Neuhaus, D. (2004) Solution structure of subunit F(6) from the peripheral stalk region of ATP synthase from bovine heart mitochondria, *J. Mol. Biol. 342*, 593−603.
20. Rubinstein, J. L., Dickson, V. K., Runswick, M. J., and Walker, J. E. (2005) ATP synthase from *Saccharomyces cerevisiae*: location of subunit h in the peripheral stalk region, *J. Mol. Biol. 345*, 513−520.
21. Walker, J. E., and Kane Dickson, V. (2006) The peripheral stalk of the mitochondrial ATP synthase, *Biochim Biophys Acta* (in press).
22. Vaillier, J., Arselin, G., Graves, P. V., Camougrand, N., and Velours, J. (1999) Isolation of supernumerary yeast ATP synthase subunits e and i. Characterization of subunit i and disruption of its structural gene ATP18, *J. Biol. Chem. 274*, 543−548.
23. Paumard, P., Vaillier, J., Napias, C., Arselin, G., Brèthes, D., Graves, P. V., and Velours, J. (2000) Environmental study of subunit i, a F(o) component of the yeast ATP synthase, *Biochemistry 39*, 4199−4205.
24. Paumard, P., Arselin, G., Vaillier, J., Chaignepain, S., Bathany, K., Schmitter, J. M., Brèthes, D., and Velours, J. (2002) Two ATP synthases can be linked through subunits i in the inner mitochondrial membrane of *Saccharomyces cerevisiae*, *Biochemistry 41*, 10390−10396.
25. Paul, M. F., Guerin, B., and Velours, J. (1992) The C-terminal region of subunit 4 (subunit b) is essential for assembly of the F_o portion of yeast mitochondrial ATP synthase, *Eur. J. Biochem. 205*, 163−172.
26. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction, *Gene 77*, 51−59.
27. Guldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J. H. (1996) A new efficient gene disruption cassette for repeated use in budding yeast, *Nucleic Acids Res. 24*, 2519−2524.
28. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations, *J. Bacteriol. 153*, 163−168.
29. Guerin, B., Labbe, P., and Somlo, M. (1979) Preparation of yeast mitochondria (*Saccharomyces cerevisiae*) with good P/O and respiratory control ratios, *Methods Enzymol. 55*, 149−159.
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J. Biol. Chem. 193*, 265−275.
31. Schägger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal. Biochem. 166*, 368−379.
32. Ansorge, W. (1983) Fast visualisation of protein bands by impregnation in potassium permanganate and silver nitrate, in *Electrophoresis 82* (Stathakos, D., Ed.) pp 235−242, Walter de Gruyter, Berlin.
33. Velours, J., Arselin de Chateaubodeau, G., Galante, M., and Guerin, B. (1987) Subunit 4 of ATP synthase (F_oF_1) from yeast mitochondria. Purification, amino-acid composition and partial N-terminal sequence, *Eur. J. Biochem. 164*, 579−584.
34. Schägger, H. (2003) Blue native electrophoresis, in *Membrane Purification and Crystallization: a practical* guide (Hunte, C., von Jagow, G., and Schägger, H., Eds.) 2nd ed., pp 105−142, Elsevier, Amsterdam.
35. Schägger, H., and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form, *Anal. Biochem. 199*, 223−231.
36. Grandier-Vazeille, X., and Guerin, M. (1996) Separation by blue native and colorless native polyacrylamide gel electrophoresis of the oxidative phosphorylation complexes of yeast mitochondria solubilized by different detergents: specific staining of the different complexes, *Anal. Biochem. 242*, 248−254.
37. Valiyaveetil, F. I., and Fillingame, R. H. (1998) Transmembrane topography of subunit a in the *Escherichia coli* F_1F_o ATP synthase, *J. Biol. Chem. 273*, 16241−16247.
38. Arselin, G., Giraud, M. F., Dautant, A., Vaillier, J., Brèthes, D., Coulary-Salin, B., Schaeffer, J., and Velours, J. (2003) The GxxxG motif of the transmembrane domain of subunit e is involved in the dimerization/oligomerization of the yeast ATP synthase complex in the mitochondrial membrane, *Eur. J. Biochem. 270*, 1875−1884.
39. Everard-Gigot, V., Dunn, C. D., Dolan, B. M., Brunner, S., Jensen, R. E., and Stuart, R. A. (2005) Functional analysis of subunit e of the F_1F_o-ATP synthase of the yeast *Saccharomyces cerevisiae*: importance of the N-terminal membrane anchor region, *Eukaryotic Cell 4*, 346−355.
40. Bustos, D. M., and Velours, J. (2005) The modification of the conserved GXXXG motif of the membrane-spanning segment of subunit g destabilizes the supramolecular species of yeast ATP synthase, *J. Biol. Chem. 280*, 29004−29010.

41. Saddar, S., and Stuart, R. A. (2005) The yeast F(1)F(0)-ATP synthase: analysis of the molecular organization of subunit g and the importance of a conserved GXXXG motif, *J. Biol. Chem. 280*, 24435−24442.
42. Rexroth, S., Meyer Zu Tittingdorf, J. M., Schwassmann, H. J., Krause, F., Seelert, H., and Dencher, N. A. (2004) Dimeric H^+-ATP synthase in the chloroplast of *Chlamydomonas reinhardti*i, *Biochim. Biophys. Acta 1658*, 202−211.
43. Minauro-Sanmiguel, F., Wilkens, S., and Garcia, J. J. (2005) Structure of dimeric mitochondrial ATP synthase: novel F_o bridging features and the structural basis of mitochondrial cristae biogenesis, *Proc. Natl. Acad. Sci. U.S.A. 102*, 12356−12358.
44. Dudkina, N. V., Heinemeyer, J., Keegstra, W., Boekema, E. J., and Braun, H. P. (2005) Structure of dimeric ATP synthase from mitochondria: An angular association of monomers induces the strong curvature of the inner membrane, *FEBS Lett. 579*, 5769−5772.

BI0601407