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Two ATP Synthases Can Be Linked through Subunits *i* in the Inner Mitochondrial Membrane of *Saccharomyces cerevisiae*†

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ABSTRACT: Cross-linking experiments showed that the supernumerary subunit *i* is close to the interface between two ATP synthases. These data were used to demonstrate the presence of ATP synthase dimers in the inner mitochondrial membrane of *Saccharomyces cerevisiae*. A cysteine residue was introduced into the inter-membrane space located C-terminal part of subunit *i*. Cross-linking experiments revealed a dimerization of subunit *i*. This cross-linking occurred only with the dimeric form of the enzyme after incubating intact mitochondria with a bis-maleimide reagent, thus indicating an inter-ATP synthase cross-linking, whereas the monomeric form of the enzyme exhibited only an intra-ATP synthase cross-linking with subunit 6, another component of the membranous domain of the ATP synthase.

The F_0F_1-ATP synthase is a molecular rotary motor that is responsible for the aerobic synthesis of ATP. It exhibits a headpiece (catalytic sector), a basepiece (membrane sector), and two connecting stalks. The sector F_1 containing the headpiece is a water-soluble unit retaining the ability to hydrolyze ATP when in a soluble form. F_0 is embedded in the membrane and is mainly composed of hydrophobic subunits forming a specific proton conducting pathway. When the F_1 and F_0 sectors are coupled, the enzyme functions as a reversible H^+-transporting ATPase or ATP synthase (*1*−*3*). The two connecting stalks are constituted of components from both F_1 and F_0. The first stalk is the rotor part of the enzyme. The second stalk, which is part of the stator, relays F_1 and hydrophobic membranous components of the enzyme. High-resolution X-ray crystallographic data have led to solving the structure of the F_1 (*4*−*7*) from different sources. Recently, Stock et al., (*8*) reported the 3.9 Å resolution X-ray diffraction structure of the *Saccharomyces cerevisiae* F_1 associated with the c-ring oligomer.

In *E. coli*, F_0 is composed of subunits *a*, *b*, and *c*. The mitochondrial F_0 of mammalian is composed of 10 different subunits (*9*). The same 10 components have been identified in the *S. cerevisiae* enzyme (*10*−*13*). Among them, the associated proteins *e* and *g* are not only involved in the dimerization of the yeast enzyme (*12*) but also in mitochondrial morphology (*14*). Furthermore, subunits *k* and *i*/*j* have also been identified in the yeast complex (*15*, *16*). Subunit *i* (also named *j*) is encoded by the nuclear gene ATP18. Subunit *i* is 59 amino acids long and corresponds to a calculated mass of 6687 Da. It is an integral inner membrane protein which spans the membrane once with a $N_{in}-C_{out}$ orientation (*15*). Although displaying a proton leak at the ATP synthase level, the null mutant ΔATP18 is able to grow on nonfermentable medium, thus indicating that the protein is not essential (*16*). The environment of subunit *i* was studied by the combination of cysteine-generated mutants, chemical modification reagents, and cross-linking reagents. That study reported that an interaction occurred between the unique membrane-spanning segment of subunit *i* and the first transmembranous α-helix of subunit 6 (the homologous protein to the mammalian or prokaryotic *a*-subunit), which is a main component of the proton channel. In addition, it revealed a relationship between subunit *i* and subunits *d*, *f*, and *g*, all of them being other components of the F_0 sector (*17*).

The yeast ATP synthase has been shown to exist as both dimeric and oligomeric forms in Triton X-100 and digitonin extracts (*12*, *14*). Subunits *e*, *g*, and *b* are important for the dimerization/oligomerization of the yeast ATP synthase, thus showing the involvement of F_0 in this process (*12*, *18*). The purpose of the present work was to provide information about the presence of the dimeric form of the ATP synthase in the inner mitochondrial membrane. Here, we show from cross-linking experiments that subunit *i*, another component of F_0 whose stoichiometry is one per complex, is able to dimerize, thus indicating that subunit *i* is close to the interface between two ATP synthase monomers and that F_1-F_0 dimers are present in the yeast inner mitochondrial membrane.

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[1] Abbreviations: BN-PAGE, blue native polyacrylamide slab gel electrophoresis; F_0 and F_1, integral membrane and peripheral portions of ATP synthase; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; NEM, *N*-ethylmaleimide; Ni−NTA, nickel nitrilotriacetic acid; oPD, *N*,*N*′-(1,2-phenylene)dimaleimide; pPD, *N*,*N*′-(1,4-phenylene)dimaleimide; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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EXPERIMENTAL PROCEDURES

Materials. *N,N′*-(1,2-phenylene)dimaleimide and digitonin were from Sigma. *N,N′*-(1,4-phenylene)dimaleimide was from Fluka. Oligonucleotides were purchased from MWG-BIOTECH. All other reagents were of reagent grade quality.

Strains and Nucleic Acid Techniques. The *Saccharomyces cerevisiae* strain D273−10B/A/H/U (MATα, *met6*, *his3*, *ura3*) was the wild-type strain (*19*). The null mutant ΔATP18 was described previously (*16*). The yeast mutants were named as (wild-type residue)(residue number)(mutant residue), where the residues were given a single-letter code. The strains containing modified versions of subunit *i* were obtained after insertion at the *his3* locus of the null mutant ΔATP18 of the integrative plasmid pRS303 cleaved at the unique *Pst*1 site and containing the 1402 bp *Eco*RI-*Bam*HI DNA fragment bearing the mutated versions of ATP18 gene. Mutagenesis was performed as described in ref *22*. Single-stranded DNA of the phagemid pDR1ATP18 was prepared from *E. coli* JM109 cultures containing the recombinant phagemid and the helper phage R408. This served as a template for mutagenesis by using the phosphorylated mutagenic oligonucleotides, the phosphorylated AmpR-oligonucleotide (*20*), and T7 DNA polymerase. Mutations were confirmed by DNA sequencing (*21*). The LiCl method (*22*) was used to transform the null mutant ΔATP18 by the recombinant integrative plasmid. Transformants were selected and subcloned on minimal medium containing methionine and glucose as carbon source. Correct integration was verified by polymerase chain reaction analysis on yeast clones. The K51C−$(His)_6$ mutant was constructed according to the following strategy. A *Aat*II restriction site was introduced into the ATP18 gene by site-directed mutagenesis of the phagemid pDR1ATP18 with the oligonucleotide 5′-GTTTGTCCTAGCATTGACGTCTCAACTTCCACAAAC-3′. This removed the stop codon of the ATP18 gene. Two complementary oligonucleotides 5′-CTCACCATCACCATCACCATTAAGATCTGACGT-3′ and 5′-CAGATCTTAATGGTGATGGTGATGGTGAGACGT-3′ encoding a $(His)_6$ sequence and bearing a *Bgl*II restriction site were annealed and ligated into the *Aat*II linearized pDR1ATP18 phagemid. The *Bgl*II site was used to screen the clones containing the DNA fragment. This resulted in a subunit *i* whose C-terminus showed a glutamic residue in position 59 instead of an aspartic residue followed by the additional sequence TSHHHHHH. The 1431 bp *Bam*H1-*Eco*RI DNA fragment containing the modified ATP18 gene was cloned in the pRS303 plasmid. The mutations K51C and K29C were introduced by the PCR mutagenesis procedure (*23*).

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. Mitochondria were prepared as described previously (*24*) 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 Lowry et al., (*25*) in the presence of 5% SDS with bovine serum albumin as a standard protein. ATPase specific activity was measured at pH 8.4 as in ref *26*.

Cross-linking experiments. For cross-linking experiments with CuCl2, oPD, and pPD, mitochondria were washed twice with 0.6 M mannitol, 0.5 mM EGTA, 50 mM HEPES, pH 7.0, and suspended in the same buffer at a protein concentration of 10 mg/mL. This suspension was incubated in the absence or in the presence of 1.5 mM CuCl2 or 300 μM of either oPD or pPD for 1 h at 4 °C. The control experiments were incubated for the same duration with 5 mM NEM. The disulfide bond formation by CuCl2 was stopped by 10 mM EDTA and 5 mM NEM. Reactions involving oPD and pPD were stopped by 5 mM NEM addition, followed 15 min later by addition of 25 mM of 2-mercaptoethanol. The mitochondrial Triton X-100 extracts were prepared as in ref *17* and incubated with pPD as above.

Cross-Linked Protein Identification Procedure. Purification of $(His)_6$-tagged proteins was done as in ref *27*. The purified proteins were treated as in ref *28* and submitted to SDS-gel electrophoresis, and the slab gel was stained with Amido Black. The selected bands were cut and destained, and the proteins they contained were cleaved in the gel slices at 37 °C with either trypsin or endoproteinase Lys-C for 16 h (*29*). The resulting peptides were extracted and concentrated in a vacuum centrifuge (*30*).

Mass Spectrometry. Peptides extracted from gels were purified and concentrated by means of C_{18} Zip Tips (Millipore) and mixed 1:1 with matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile, 0.1% aqueous trifluoroacetic acid). Analysis was performed with a Bruker Reflex III instrument functioning in the reflection mode for mass measurements (external calibration) and post-source decay analysis of selected precursors.

Electrophoretic Analyses and Western Blot Analyses. SDS-gel electrophoresis was done according to Schägger and Von Jagow (*31*). Western blot analyses were described previously (*32*). Polyclonal antibodies were used at a 1:10 000 dilution. Evaluation of the apparent molecular masses of cross-linked products was done by using molecular masses of subunits *i*, *f*, and *d* as reported previously (*17*). The polyclonal antibodies raised against subunit *i* were directed against a C-terminal peptide containing amino acid residues 41−54.

BN-PAGE experiments were done as described in (*33*, *34*). Mitochondria (1 mg of protein) were incubated for 30 min at 4 °C with 0.1 mL of digitonin at a digitonin/protein ratio of 2 g/g. The extracts were centrifuged at 4 °C for 15 min at 40 000*g*, and aliquots of the supernatant (40 μL) were immediately loaded on the top of a 3−13% polyacrylamide slab gel. After electrophoresis, the gel was divided into two parts. The first part of the gel was incubated in a solution of 5 mM ATP, 5 mM MgCl2, 0.05% lead acetate, 50 mM glycine−NaOH pH 8.4 to reveal the ATPase activity (*35*, *36*). From the second part of the gel, protein bands containing the monomeric and dimeric form of the ATP synthase were cut and submitted to SDS-gel electrophoresis. The resulting gel was then blotted onto nitrocellulose membranes (Membrane Protean BA83, 0.2 μm from Schleicher et Schuell) that were probed with polyclonal antibodies raised against subunit *i*.

RESULTS

The additional subunit *i* is a component of the yeast F_0 located in the periphery of the ATP synthase. In a previous report, a topological analysis with the bifunctional reagent dithiobis(succinimidyl propionate) revealed the proximity of subunit *i* and structural components of the ATP synthase (*17*).

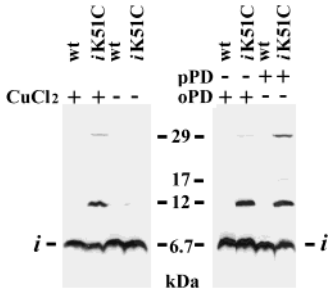

FIGURE 1: Cross-linking of the C-terminal part of subunit *i* with other proteins having a cysteine residue located in the intermembrane space. Wild-type and *i*K51C mitochondria (0.5 mg of protein) were incubated with 1.5 mM CuCl2 or with either 300 μM of oPD or 300 μM of pPD as described in the method section, and samples (50 μg of protein) were analyzed by Western blot. The blots were incubated with polyclonal antibodies raised against subunit *i*. wt = wild-type mitochondria.

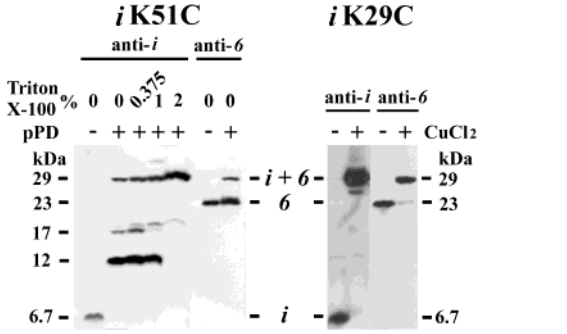

FIGURE 2: Decrease in the formation by pPD of the 12 kDa adduct in the presence of high Triton X-100 concentrations. Left part of the figure: mitochondria and Triton X-100 extracts of the *i*K51C strain were incubated with 300 μM of pPD. Right part of the figure: the 0.375% Triton X-100 extract of *i*K29C mitochondria was incubated in the presence or absence of 1.5 mM CuCl2. Samples (30 μg of protein) were analyzed by Western blot. The blots were incubated with polyclonal antibodies raised against subunits *i* and 6.

Each of the six lysine residues of subunit *i* was substituted one by one for cysteine residues to obtain information on the environment of the subunit. We showed that the C-terminal part of the protein, which is located in the intermembrane space, is close to the N-terminus of subunit 6 (an essential component of the proton channel), since adducts were obtained by disulfide bond formation between Cys^{23} of subunit 6 and either position 29 or position 54 of subunit *i*. In addition, a 12 kDa adduct was obtained by oxidation with *i*K54C mitochondria, corresponding to a cross-linking of subunit *i* (6687 Da) with a nonidentified product having an apparent molecular mass of 5.5 kDa (*17*). This cross-linking product was lost in the yeast strain having a $(His)_6$ sequence at the C-terminus of subunit *i*K54C. As a consequence, we looked for other positions in the C-terminal part of subunit *i* which could lead to the 12 kDa adduct formation. In this work, the bis-maleimide cross-linking reagents oPD and pPD (average S−S distances of 9.39 ± 0.47 and 11.13 ± 0.52 Å, respectively; *37*) were used to obtain nonreversible cross-linked products.

Identification of Proteins Located in the Vicinity of Position 51 of Subunit i. Mitochondria isolated from the *i*K51C strain like those of the *i*K54C strain were able to produce adducts having relative molecular masses of 12 and 29 kDa. These adducts were obtained either by disulfide bond formation or by alkylation with the bis-maleimide reagents oPD and pPD (Figure 1). The amount of the 29 kDa adduct obtained with pPD was higher than that obtained upon incubation with oPD. This band was identified as a cross-linking product between subunit *i* (6.7 kDa) and subunit 6 (relative molecular mass of 23 kDa), thus involving the unique Cys^{23} of subunit 6 and Cys^{51} of subunit *i*, a result similar to that previously obtained from position 54 (*17*). In addition, a faint band at 17 kDa was found upon incubation with pPD.

It has been reported that subunit *i* also forms cross-linked products with proteins that are loosely bound to the yeast enzyme (*17*). This was investigated by cross-linking experiments performed in the presence of high Triton X-100 concentrations (Figure 2). Analysis of *i*K51C Triton X-100 extracts revealed a strong increase in the amount of the 29 kDa adduct upon increasing the detergent concentration. The presence of this band, which was identified by western blot as a *i* + 6 heterodimer, indicates the strong interaction of these subunits under these experimental conditions. This was not the case with the 12 and 17 kDa adducts, which disappeared when the Triton X-100 concentration was increased, since the 12 kDa adduct was absent in the presence of 2% Triton X-100, whereas the 17 kDa adduct was lost with 1% of Triton X-100. This result indicates that detergent addition prevented the formation of these cross-links, either by removing the partner or by increasing the distance between them. The consequence of this lack of reactivity with the labile partners was an increase in the amount of *i* + 6 heterodimer, two subunits which are close. Depending on the location of the cysteine residue in the C-terminal part of subunit *i*, the yield of cross-linked reaction between the subunits *i* and 6 varied. The highest yield in adduct was obtained with the *i*K29C mutant (Figure 2). In this mutant, a cysteine residue was introduced at position 29, a position which is predicted at the end of the unique transmembrane-segment of subunit *i* and which is close to Cys^{23} of subunit 6 (*17*). Upon oxidation of the *i*K29C Triton X-100 extract, the entire subunit 6 band migrated at 29 kDa, and the entire *i*-subunit was also converted to the 29 kDa band, so it appears that the two proteins have the same stoichiometry. A stoichiometry of one subunit 6 per ATP synthase had previously been established (*38*). As a consequence, there is only one subunit *i* per complex. This result is in agreement with that of Arnold et al. (*12*), who obtained a stoichiometry of 0.87 by densitometric quantification of Coomassie-stained gels.

The identification of proteins close to subunit *i* and involved in the 12 and 17 kDa adducts was unsuccessful when we used our set of polyclonal antibodies raised against ATP synthase subunits. Therefore, the purification of oPD-dependent cross-linked products was performed according to the following strategy. A $(His)_6$ sequence was placed at the C-terminus of subunit *i*K51C. The addition of this additional sequence altered neither cell growth with lactate or glycerol as carbon source nor mitochondrial ATPase sensitivity toward F_0 inhibitors (Table 1). Intact mitochondria isolated from *i*K51C and *i*K51C−$(His)_6$ strains were incubated with either oPD or pPD and analyzed by Western blot (Figure 3). The mutant *i*K51C−$(His)_6$ displayed an increase in the mass of subunit *i* which was the consequence of the additional $(His)_6$ sequence. Mass increases were also found in the molecular mass of the corresponding adducts. As

Table 1: Generation Time of Yeast Strains and ATPase Activities of Yeast Mitochondria[a]

strains	doubling time (min)	ATPase activity [μmol of Pi min-1 (mg of protein)-1] no addition	oligomycin
wild-type control	165	6.46 ± 0.27	0.48 ± 0.05
*i*K51C	168	5.52 ± 0.22	0.50 ± 0.20
*i*K51C−(His)$_6$	159	6.31 ± 0.14	0.22 ± 0.04

[a] Growth was monitored by turbidimetry at 600 nm. The growth rate was calculated in the logarithmic growth phase over a 10 h period. Yeast cells were grown at 28 °C with 2% lactate as the carbon source. Mitochondria were isolated. ATPase assays were performed in triplicate at 30 °C in the presence and in the absence of oligomycin (6 μg/mL).

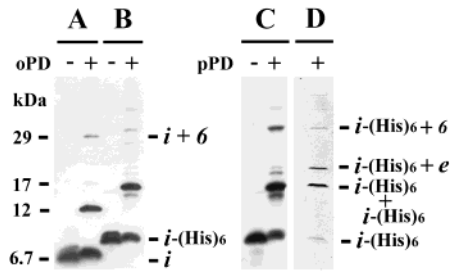

FIGURE 3: Purification of cross-linked products involving Cys[51] of subunit *i*. Mitochondria isolated from mutant strains *i*K51C (A) and *i*K51C−(His)$_6$ (B−D) were incubated with either 300 μM oPD or 300 μM pPD. (A−C) samples (30 μg of protein) were analyzed by Western blot. The blots were incubated with polyclonal antibodies raised against subunit *i*. (D) pPD-treated *i*K51C−(His)$_6$ mitochondria (50 mg of protein) were solubilized, and subunit *i* and adducts were purified by Ni−NTA chromatography followed by SDS-gel electrophoresis. The slab gel was stained with Amido Black, and the bands were cut for proteolysis and mass spectrometry analyses. The identification of subunit *i* partners was obtained from Table 2.

shown in Figure 1, incubation of mitochondria with the bis-maleimide reagent pPD resulted in a high amount of the *i* + 6 adduct. Four main bands were revealed by the antibody raised against subunit *i* (Figure 3C). The proteins linked to subunit *i*K51C−(His)$_6$ were identified as follows. Subunit *i* and adducts were purified from mitochondrial extracts by Ni−NTA chromatography. The proteins were separated by SDS-gel electrophoresis, and the gel was stained with Amido Black (Figure 3D). The pieces of gel containing the stained bands were destained and incubated with either trypsin or endoproteinase Lys-C. The resulting peptides were extracted and analyzed by MALDI-MS (see Experimental procedures). Table 2 shows the masses of peptides obtained by tryptic cleavage of the so-called 6.7, 12, 17, and 29 kDa bands. Four tryptic peptides and three endoproteinase Lyc-C peptides (not shown) confirmed that the 6.7 kDa band was subunit *i*K51C−(His)$_6$ (Table 2). Fragmentation of the 12 kDa band resulted in five tryptic peptides (Table 2) and three endoproteinase Lyc-C peptides (not shown) belonging to the unique subunit *i*K51C−(His)$_6$. As a consequence, this band was identified as a dimer of subunit *i*. From the 17 kDa band, three tryptic peptides of subunit *i*K51C−(His)$_6$ and three other peptides of subunit *e* were identified. The formal identification of the *e*-subunit was performed by post-source decay analysis of the parent ion at *m*/*z* 2162.73 ([M+H]+ monoisotopic mass), which is likely to correspond to the peptide [63−81] of subunit *e* (calculated $[M + H]^+$ monoisotopic mass is 2163.00 Da). The peptide was identified by means of 25 fragment ions, including a series of contiguous C-terminal ions (y type) from 1 to 7 residues long. From the 29 kDa band, it was apparent that four tryptic peptides belonged to subunit *i*K51C−(His)$_6$ and one to subunit 6. The formal identification of subunit 6 was performed by analyzing post-source decay fragment ions originating from the precursor ion at *m*/*z* 1104.51, corresponding to the peptide [1−9] of subunit 6 (calculated $[M + H]^+$ monoisotopic mass is 1104.57 Da). Two series of C-terminal ions (y1 to y5) and N-terminal ions (b2 to b7), together with internal ions, confirmed that this peptide belongs to subunit 6.

ATP Synthase Dimers Are Present in the Yeast Inner Mitochondrial Membrane. Since the stoichiometry of subunit

Table 2: Mass Spectrometric Analyses of Tryptic Peptides of *i*K51C−(His)$_6$ Adducts[a]

sample	[M + H]+ *m*/*z* measured	calcd	subunit	residues	peptide sequence
6.7 kDa band	815.61	815.50	*i*K51C−(His)$_6$	[5−11]	FPTPILK
	890.50	890.44	*i*K51C−(His)$_6$	[39−45]	EFINDPR
	971.60	971.60	*i*K51C−(His)$_6$	[4−11]	RFPTPILK
	1632.70	1632.74	*i*K51C−(His)$_6$	[55−67]	FVEVETSHHHHHH
12 kDa band	815.61	815.50	*i*K51C−(His)$_6$	[5−11]	FPTPILK
	890.43	890.44	*i*K51C−(His)$_6$	[39−45]	EFINDPR
	971.55	971.60	*i*K51C−(His)$_6$	[4−11]	RFPTPILK
	1632.62	1632.74	*i*K51C−(His)$_6$	[55−67]	FVEVETSHHHHHH
	2055.86	2056.00	*i*K51C−(His)$_6$	[12−29]	VYWPFFVAGAAVYYGMSK
17 kDa band	890.34	890.44	*i*K51C−(His)$_6$	[39−45]	EFINDPR
	971.47	971.60	*i*K51C−(His)$_6$	[4−11]	RFPTPILK
	1632.50	1632.74	*i*K51C−(His)$_6$	[55−67]	FVEVETSHHHHHH
	1085.46	1085.66	e	[82−91]	RFPTPILK
	1447.55	1447.77	e	[8−20]	YSALGLGLFFGFR
	2162.73	2163.00	e	[63−81]	DVPANASFNLEDPNIDFER
29 kDa band	815.51	815.50	*i*K51C−(His)$_6$	[5−11]	FPTPILK
	890.42	890.44	*i*K51C−(His)$_6$	[39−45]	EFINDPR
	971.54	971.60	*i*K51C−(His)$_6$	[4−11]	RFPTPILK
	1632.57	1632.74	*i*K51C−(His)$_6$	[55−67]	FVEVETSHHHHHH
	1104.51	1104.57	6	[1−9]	SPLDQFEIR

[a] The bands corresponding to adducts involving *i*K51C−(His)$_6$ were purified by SDS-gel electrophoresis after Ni−NTA chromatography. They are referred as to the apparent molecular mass of the corresponding adduct in the *i*K51C strain.

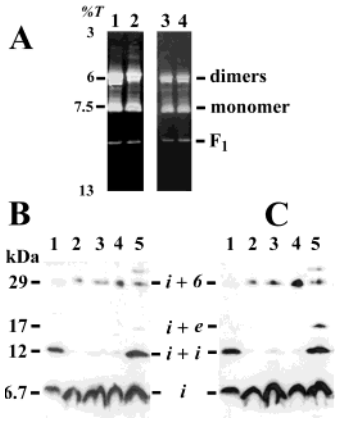

FIGURE 4: ATP synthase dimers are present in the yeast mitochondrial inner membrane. (A) Lanes 1 and 2: *i*K51C mitochondria were incubated with either 5 mM NEM (lane 1) or 300 μM oPD (lane 2) and were extracted with digitonin (digitonin/protein ratio of 2 g/g). Lanes 3 and 4: *i*K51C mitochondrial digitonin extracts (digitonin/protein ratio of 2 g/g) were incubated with either 5 mM NEM (lane 3) or 300 μM oPD (lane 4). The digitonin extracts were centrifuged and the supernatants (corresponding to 200 μg of protein) were loaded onto the top of a 3−13% polyacrylamide slab gel. The position of F_1 and of monomeric and dimeric forms of the ATP synthase was revealed in the slab gel by the ATPase activity. The gel slices containing the dimeric and monomeric forms of the ATP synthase were cut from the native gel, incubated with 1% SDS for 1 h at room temperature, submitted to SDS-gel electrophoresis, and analyzed by Western blot. (B) Western blot analysis of monomeric and dimeric forms isolated by BN-PAGE from NEM or oPD treated *i*K51C mitochondria. (C) Western blot analysis of monomeric and dimeric forms isolated by BN-PAGE from NEM or oPD treated *i*K51C mitochondrial digitonin extracts. The blots were incubated with polyclonal antibodies raised against subunit *i*. Lanes 1: *i*K51C mitochondria (50 μg of protein) treated with 300 μM of oPD and dissociated with SDS (control experiment). Lanes 2 and 4: monomeric forms of the ATP synthase isolated from samples treated with NEM and oPD, respectively. Lanes 3 and 5: dimeric form of the ATP synthase isolated from samples treated with NEM and oPD, respectively. *%T* = acrylamide concentration.

i is 1, the above-mentioned dimerization of subunit *i* indicates the proximity of two subunits *i*, each belonging to distinct ATP synthases. This result was reinforced by the lack of the $i + i$ adduct in the presence of 2% Triton X-100 (Figure 2), conditions known to dissociate ATP synthase dimers (*12*).

$i + i$ dimerization was used to demonstrate the existence of ATP synthase dimers in the inner mitochondrial membrane. Intact mitochondria were incubated with oPD to cross-link subunits *i*, whereas control mitochondria were incubated with NEM to block Cys^{51}. NEM treatment prevented the dimerization of subunit *i* via disulfide bond formation. After alkylation, mitochondria were solubilized by digitonin addition with a digitonin/protein ratio of 2 g/g, conditions leading to monomeric and dimeric forms of the yeast ATP synthase, as shown by the BN-PAGE experiment in Figure 4A (lanes 1 and 2) and ref *12*. In these conditions the monomeric and dimeric forms of the ATP synthase migrated at acrylamide concentrations of 7.5 and 6%, respectively (*14*). Slices of gel containing the two forms of the enzyme were analyzed by SDS-gel electrophoresis and by Western blot (Figure 4B). The monomeric form of the enzyme displayed a $i + 6$ adduct (Figure 4B, lane 4) resulting from the intra-ATP synthase cross-linking between Cys^{51} of subunit *i* and the unique Cys^{23} of subunit 6, a target also located in the intermembrane space. This result is in agreement with the existence of the $i + 6$ adduct in the presence of 2% Triton X-100 (Figure 2), conditions which lead to the monomeric form of the ATP synthase only (*12*). The $i + i$ dimer was found only in the piece of gel containing the dimeric form of yeast ATP synthase (Figure 4B, lane 5). This result is clearly in favor of an inter-ATP synthase cross-linking in the membrane between two subunits *i*.

The cross-linking experiments with oPD were also performed on digitonin extract of *i*K51C mitochondria in conditions where monomeric and dimeric forms of the ATP synthases were solubilized (Figure 4A, lanes 3 and 4). Then the two forms of the ATP synthase were separated by BN-PAGE. The $i + i$ dimer was detected only in the piece of gel containing the dimeric form of the ATP synthase (Figure 4C, lane 5), which resulted from an inter-ATP synthase cross-linking. The 17 kDa band, previously identified as an $i + e$ heterodimer, was also detected in the dimeric form of the ATP synthase. Again, the monomeric form of the enzyme displayed an intense $i + 6$ adduct resulting from the intra-ATP synthase cross-linking (Figure 4C, lane 4). Traces of $i + 6$ adducts were also observed in control samples (Figure 4B,C, lanes 2 and 3). Despite the treatment of control samples by 5 mM NEM incubation, disulfide bond formation occurred between subunits *i* and 6, a fact very likely indicating the existence of such cross-linking before maleimide treatment.

The data reported above involved the bis-maleimide reagent oPD. Indeed, incubation of either *i*K51C mitochondria or *i*K51C digitonin extract with pPD resulted in similar results to those obtained with oPD (presence of a subunit *i* dimer in the dimeric form of the ATP synthase and only a $i + 6$ adduct in the monomeric form of the ATP synthase, not shown). In addition, an intense $i + e$ adduct in the dimeric form of the ATP synthase was also obtained upon incubation of *i*K51C digitonin extract with pPD (not shown).

DISCUSSION

Subunit *i* is an additional protein of the yeast ATP synthase which has not been found in higher eukaryotes so far. Homologous proteins are likely to exist since open reading frames encoding this protein have been identified in *Schizosaccharomyces pombe* (EMBL accession number Z99753), *Neurospora crassa* (Gene bank, accession number AI329387), and *Caenorhabditis elegans* (Gene bank accession number AF067943.1 22834.22974). With a unique transmembrane segment and an $N_{in}-C_{out}$ orientation, subunit *i* displays a short hydrophilic segment in the matrix (amino acid residues 1−6) and a C-terminal hydrophilic segment in the intermembrane space (amino acid residues 29−59).

Environment of the C-Terminal Part of Subunit i in the Intermembrane Space. The environment of the C-terminal part of subunit *i* has previously been investigated by engineered cysteine mutants and cross-linking experiments. Unidentified partners that are loosely bound to the yeast ATP synthase led us to propose that subunit *i* is located in the periphery of the enzyme (*17*). To identify its unknown close partners, we used a yeast mutant having a unique cysteine residue at position 51 of subunit *i* and whose C-terminal part contained an additional $(His)_6$ sequence. From position 51, three cross-linking products reacted with the anti-*i* antibody: (i) a 12 kDa band already observed with mutant *i*K54C (*17*); (ii) a faint band at 17 kDa; and (iii) a 29 kDa band

already identified as an $i + 6$ adduct and also obtained from positions 29 and 54 (*17*). After isolation of cross-linked products and mass spectrometry analyses of peptide digests, it appeared that the 12 kDa and 17 kDa adducts were a dimer of subunit *i* and a heterodimer of subunits *i* and *e*, respectively. As shown in Figure 2, the 17 kDa adduct was not present in cross-linked 1% (or more) Triton X-100 extracts. This can easily be explained by the removal of subunit *e* from the ATP synthase upon incubation with Triton X-100 concentrations higher than 0.375%, as previously described (*16*).

Incubation of the digitonin extract with oPD led to a higher amount of $i + e$ adduct than with intact mitochondria (Figure 4B,C, lanes 5). This result was also observed with pPD in the presence of 0.375% Triton X-100 (Figure 2). Such a cross-link likely involved Cys^{51} of subunit *i* and the unique endogenous cysteine residue of subunit *e* (Cys^{28}). This reflects a higher accessibility of Cys^{28} of subunit *e* to the cross-linking reagent in the solubilized form of the ATP synthase than in the membrane. This is in agreement with the predicted position of Cys^{28}, which is located at the end of the unique transmembranous domain on the intermembrane space side. From the above data, no definitive conclusions regarding the inter- or intra-ATP synthase behavior of the $e + i$ adduct can be drawn. Subunit *e* is known to be involved in the dimerization of the enzyme and was found only in the dimeric form of the ATP synthases (not shown and ref *12*). As a result, the absence of the $i + e$ adduct in the monomeric form of the ATP synthase obtained after cross-linking of the digitonin extract (Figure 4C, lane 4) reflects the absence of subunit *e* in this form of the enzyme. On the other hand, the low intensity of the $i + e$ adduct in the dimeric form of the ATP synthase after incubation of mitochondrial membranes with oPD precludes any conclusion about the existence of the $i + e$ heterodimer in the monomeric form of the ATP synthase obtained after digitonin extraction of cross-linked mitochondria.

Dimerization of Subunit i *in the Dimeric Form of the ATP Synthase.* The formation of an $i + i$ dimer in the membrane is the major finding of this paper. Since the stoichiometry of subunit *i* is one, it demonstrates the close proximity of two subunits *i* in the dimeric form of the ATP synthase. Arnold et al. (*12*) have clearly shown the presence of ATP synthase dimers in Triton X-100 and digitonin extracts of yeast mitochondria. However, other forms of the enzyme have been described such as oligomeric forms, depending on the digitonin/protein ratio (*14*). The monomeric form of the ATP synthase is a third form of the enzyme, and we postulate that the monomeric form of the mitochondrial ATP synthase is due to the loss of subunit *e* or subunit *g*, resulting from either detergent incubation (high Triton X-100 or digitonin concentrations) or inactivation of the respective structural genes (*12*).

The question whether ATP synthase dimers have a physiological meaning could be solved by the evidence of cross-links at the membrane level between subunits belonging to two close ATP synthases and located at the interface between the two enzymes. Subunit *i* is one of the components located near this interface. However, subunit *i* is not essential in the dimerization of ATP synthase since the dimeric form of this enzyme was found by BN-PAGE in the mitochondrial digitonin extracts of the null mutant devoid of subunit *i* (*14*). The cross-linking of two subunits *i* upon incubation of intact mitochondria with oPD is clear evidence of the presence of ATP synthase dimers in the inner mitochondrial membrane of *S. cerevisiae*. Furthermore, the existence of the $i + i$ adduct before and after digitonin extraction is not in favor of the artifactual aggregation of two solubilized monomers to constitute the dimeric form of the ATP synthase. To be noted is the absence of the $i + i$ dimer in the monomeric form of the ATP synthase when digitonin extraction was performed after cross-linking. This indicates that detergent extraction did not "pull the $i + i$ dimer out" of the interacting enzymes since the latter was not found in the monomeric form. The $i + i$ cross-link probably stabilized the ATP synthase dimers during digitonin extraction, and the monomeric form was likely the result of the dissociation by digitonin of non cross-linked ATP synthase.

Dimer formation occurring in the membrane between monomeric forms via collisions due to brownian lateral diffusion of proteins during the cross-linking experiments was unlikely for the following reasons: (i) no cross-links with other mitochondrial complexes have been identified, (ii) the cross-links obtained after incubation of intact mitochondria were also obtained in detergent extracts which preserve the dimeric form of the ATP synthase, and (iii) the cross-linking reactions were performed at 4 °C, which decreases protein mobility in membranes. As a result, cross-linking in the mitochondrial membrane most probably freezes associations of ATP synthase preexisting in the membrane.

A consequence of the above data is that cross-linking of two ATP synthases through their F_0 part raises the possibility of two monomers interacting at this level. Another interface between ATP synthase dimers could be constituted by F_1 parts since dimers of bovine F_1 linked by IF_1 have been observed under electron microscopy (*39*, *40*). However, in yeast, ATP synthase dimer and oligomers still exist in the single mutants and double mutant whose *INH1* and *STF1* genes, encoding the IF_1 and the 9 kDa proteins, respectively, have been disrupted (Velours et al., unpublished work), thus calling into question the role of F_1 parts and IF_1 in the dimerization of ATP synthases.

The structure of the membranous part of the mitochondrial stator is unknown so far, but speculation is possible regarding the position of proteins involved in the dimerization of the ATP synthase at F_0 level. No cross-linking product involving subunit 9 (also called subunit *c*) and subunits *e*, *g*, and *i* has been described so far, thus indicating their distance from the rotor. Subunits *e*, *g*, and *i* are most probably located in the periphery of F_0 and are in interaction with subunit 6 (also called subunit *a*), which delivers protons to the *c*-oligomer. Subunits *i* and 6 are in contact, and the cross-linking of subunits *e* and *g* with subunit *i* has already been shown, thus indicating the proximity of the three latter subunits (*17*, *41*). With a mean diameter of 50−60 Å for the *c*-ring oligomer (*42*, *43*) and a mean diameter of 100 Å for F_1 (*4*), it is conceivable that the membranous parts of the components of the stator (subunits *8*, *b*, *f*, and *i*) in association with the five transmembranous segments of subunit 6 (*44*) support in their periphery subunits *e* and *g*, which are involved in the dimerization of the ATP synthase, thus allowing interaction between monomers at F_0 level without distorting the F_1 parts of each monomer.

As shown in this paper, the supramolecular organization of the components of the inner mitochondrial membrane can be studied by the combination of BN-PAGE, SDS-gel electrophoretic analyses of isolated supramolecular complexes and cross-linking experiments with engineered targets. This strategy appears to be a promising tool for identifying the areas of interaction between protein complexes.

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