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# Environmental Study of Subunit *i*, a $F_o$ Component of the Yeast ATP Synthase†

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ABSTRACT: The topology of subunit *i*, a component of the yeast $F_oF_1$-ATP synthase, was determined by the use of cysteine-substituted mutants. The $N_{in}-C_{out}$ orientation of this intrinsic subunit was confirmed by chemical modification of unique cysteine residues with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid. Near-neighbor relationships between subunit *i* and subunits *6*, *f*, *g*, and *d* were demonstrated by cross-link formation following sulfhydryl oxidation or reaction with homobifunctional and heterobifunctional reagents. Our data suggest interactions between the unique membrane-spanning segment of subunit *i* and the first transmembranous α-helix of subunit *6* and a stoichiometry of 1 subunit *i* per complex. Cross-linked products between mutant subunits *i* and proteins loosely bound to the $F_oF_1$-ATP synthase suggest that subunit *i* is located at the periphery of the enzyme and interacts with proteins of the inner mitochondrial membrane that are not involved in the structure of the yeast ATP synthase.

The $F_oF_1$-ATP synthase[1] is the major enzyme responsible for the aerobic synthesis of ATP. It exhibits a tripartite structure consisting of 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. $F_o$ is embedded in the membrane and is mainly composed of hydrophobic subunits forming a specific proton conducting pathway. The connecting stalks are constituted of components from both $F_1$ and $F_o$. When the $F_1$ and $F_o$ sectors are coupled, the enzyme functions as a reversible $H^+$-transporting ATPase or ATP synthase (*1*, *2*). The model for energy coupling by ATP synthase that has gained the most general support is the binding change mechanism (*3*). This concept has been strengthened by the establishment of the crystal structure of the major part of the bovine $F_1$ and rat $F_1$ (*4*, *5*). The affinity change of substrates and products at catalytic sites is coupled to proton transport by the rotation of the γ-subunit inside the $\alpha_3\beta_3$ oligomer (*6*−*9*). The ATP synthase thus operates as a rotary motor. In *Escherichia coli*, $F_1$ and $F_o$ are linked by a stalk which is made of subunits γ and ϵ and which constitutes a part of the rotor (*10*). Three other subunits, δ of $F_1$ and the two *b*-subunits of $F_o$, are also involved in the binding of $F_1$ to $F_o$. They form the stator, a second stalk which fixes the $\alpha_3\beta_3$ oligomer to the *a*-subunit, thus allowing rotation of the *c*-subunit oligomer together with the γ- and ϵ-subunits. (*7*, *12*). The *E. coli* ATP synthase and the bovine enzyme contain 8 and 16 different types of subunits, respectively (*13*). In the case of *Saccharomyces cerevisiae*, the $F_oF_1$-ATP synthase is composed of at least 13 different kinds of subunits involved in the structure of the enzyme; the disruption of each of their structural genes leads to a lack of assembly of the complex (*14*). Recently, the establishment of the structure of the yeast enzyme at 3.9 Å resolution revealed the structure of the main part of $F_1$ and that of the subunit *9* oligomer of $F_o$ (*15*). Low-resolution structural approaches such as reconstitution (*16*), chemical cross-linking (*17*, *18*), and disulfide bond formation experiments (*19*−*23*) are alternatives to obtain essential information on the $F_o$ organization and on the second stalk organization in the absence of crystallographic and NMR data.

We have already undertaken a topological study of the yeast $F_o$. Since the primary structure of each subunit is known, we have used a combination of cysteine-generated mutants and cross-linking reagents to examine the organization of stator subunits by Western blot analyses (*23*−*25*). The yeast $F_o$ is made of eight different components (*14*), subunits *4*, *6*, *8*, *9*, *d*, *f*, and *h* and OSCP, whose gene inactivation leads to a lack of growth on a nonfermentable carbon source. Seven additional proteins (e.g., $IF_1$, *9* kDa, *15* kDa, *i*, and *k*) have also been identified (*26*): they are associated to the yeast enzyme but are not essential to cell growth with glycerol or lactate as the carbon source. Among them, subunit *i* (*27*), also named subunit *j* (*28*), has recently been identified. It 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 (*28*). The null mutant ΔATP18 is able to grow on nonfermentable medium, thus indicating that the protein

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[1] Abbreviations: APA-Br, *p*-azidophenacyl bromide; CuP, Cu(II)−(1,10-phenanthroline)$_3$; DSP, dithiobis(succinimidyl propionate); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $F_o$ and $F_1$, integral membrane and peripheral portions of ATP synthase; OSCP, oligomycin-sensitivity-conferring protein; SDS, sodium dodecyl sulfate; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate.

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is not essential (*27*). However, the mutant cells grown on such a purely oxidative carbon source had an increased doubling time, so the absence of this additional subunit caused a significant alteration of oxidative phosphorylations. As a result, the role and the location of this subunit in the $F_o$ sector are under investigation. The environment of subunit *i* has now been studied by the combination of cysteine-generated mutants, chemical modification reagents, and cross-linking reagents. Here we report new data showing the proximity of subunit *i* and subunits *6*, *d*, *f*, and *g*, all of them being components of the $F_o$ sector.

## EXPERIMENTAL PROCEDURES

*Materials*. DSP and SMPB were from Pierce. AMDA and MPB were obtained from Molecular Probes. APA-Br was from Sigma. Oligonucleotides were purchased from MWG-BIOTECH. All other chemicals were of reagent grade quality.

*Strains and Nucleic Acid Techniques*. The *S. cerevisiae* strain D273-10B/A/H/U (MATα, *met6*, *his3*, *ura3*) was the wild-type strain (*29*). 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 integrative plasmid pRS303 containing the 1402 bp *Eco*RI−*Bam*HI DNA fragment bearing the wild-type ATP18 gene (*27*) or the mutated versions of the ATP18 gene, respectively. Mutagenesis was performed as described in ref *30*. Single-stranded DNA of the phagemid pDRATP18 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 $Amp^R$-oligonucleotide (*30*), and T7 DNA polymerase. Mutations were confirmed by DNA sequencing (*31*). The LiCl method (*32*) 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 the carbon source. Correct integration was verified by polymerase chain reaction analysis on yeast clones.

*Biochemical Procedures*. Cells were grown aerobically at 28 °C in a complete liquid medium containing 2% lactate as the carbon source (*33*) and harvested in the logarithmic growth phase. Mitochondria were prepared as described previously (*34*) and suspended in the isolation buffer (0.6 M mannitol, 2 mM EGTA, 10 mM Tris−maleate, pH 6.8). The protein concentration was determined according to Lowry et al. (*35*) in the presence of 5% SDS with bovine serum albumin as the standard protein. ATPase specific activity was measured at pH 8.4 as described in ref *36*.

*Cross-Linking Experiments*. For cross-linking experiments with DSP, mitochondria isolated from wild-type cells were washed twice with 0.6 M mannitol, 2 mM EGTA, and 50 mM triethanolamine−HCl, pH 8.0, and suspended in 22 mM triethanolamine−HCl, pH 8.0, at a protein concentration of 10 mg/mL. To this suspension was added an equal volume of 0.75% Triton X-100 (w/v), and incubation was performed for 20 min at 4 °C. After centrifugation (100000*g*, 15 min at 4 °C), the supernatant was incubated with DSP for 30 min at 27 °C. The reaction was quenched by the addition of 10 mM Tris. For cross-linking experiments with SMPB, wild-type and mutant mitochondria were washed twice with 0.6 M mannitol, 2 mM EGTA, and 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, pH 7.5, and were suspended in the same buffer at a protein concentration of 10 mg/mL. The Triton X-100 extract was prepared as above. Samples were incubated with or without 0.2 mM SMPB for 10 min at 25 °C. The reaction was stopped by addition of the electrophoresis sample buffer containing 2% 2-mercaptoethanol. Cross-linking experiments with APA-Br were performed as described previously (*23*). Disulfide bond formation was done by incubation of mitochondrial Triton X-100 extracts with either 1.5 mM Cu(II)−(1,10-phenanthroline)$_3$ (CuP) as in ref *37* or 2 mM diamide according to Gaballo et al. (*22*).

*SDS−Polyacrylamide Slab Gel Electrophoresis and Western Blot Analyses*. SDS gel electrophoresis was done according to the method of Schägger and Von Jagow (*38*). Western blot analyses were described previously (*39*). Polyclonal antibodies were used at a 1:10 000 dilution. ProBlott membranes (Applied Biosystems) were incubated with peroxidase-labeled antibodies at a dilution of 1:10 000 and revealed with the ECL reagent of Amersham International. Evaluation of the apparent molecular masses of cross-linked products was done by using molecular masses of subunits *i*, *f*, and *d* as standards. The polyclonal antibodies raised against subunit *i* were directed against a C-terminal peptide containing amino acid residues 41−54. Polyclonal antibodies raised against subunit *g* were kindly provided by Dr. R. Stuart and Dr. W. Neupert (Institut für Physiologishe Chemie der Universität Munchen, Munich, Germany).

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

| strains | doubling time (min) | ATPase activity [$\mu$mol of $P_i$ $min^{-1}$ (mg of protein)$^{-1}$] | |
|---|---|---|---|
| | | no addition | oligomycin |
| wild-type control | 161 | 6.46 ± 0.27 | 0.48 ± 0.05 |
| K3C | 181 | 5.06 ± 0.01 | 0.40 ± 0.03 |
| K29C | 188 | 8.08 ± 0.20 | 0.05 ± 0.01 |
| K54C | 166 | 4.53 ± 0.10 | 0.24 ± 0.03 |

[a] Growth was monitored by turbidimetry at 600 nm. The growth rate was calculated in the exponential 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 at 30 °C with the addition of 6 $\mu$g of oligomycin/mL where indicated. The wild-type control strain was the ΔATP18 strain complemented by the integrative vector pRS303 bearing the wild-type ATP18 gene.

## RESULTS

Subunit *i* contains a unique membrane-spanning segment. The location of the N- and C-termini was examined by using two cysteine-substituted mutants (K3C and K54C) having a unique cysteine residue in the N- and in the C-terminal parts of the protein, respectively. Growth of the mutant strains on nonfermentable medium and the oligomycin-sensitive ATPase activities were not significantly affected by these substitutions (Table 1). The accessibility of the cysteines was determined by using hydrophilic maleimide reagents as was done previously for subunits *4* and *f* (*23*, *25*). In intact mitochondria, $Cys^{54}$ of subunit *i*K54C was accessible to 4-acetamido-4′-maleimidylstilbene-2,2′disulfonic acid and

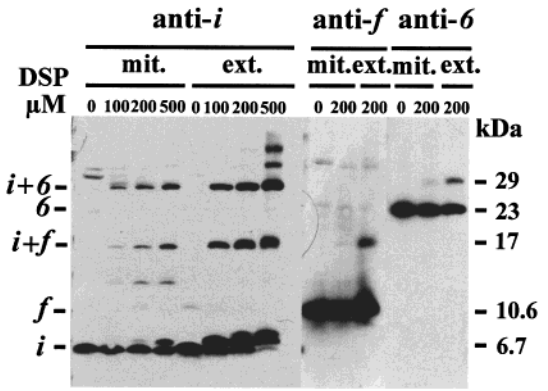


FIGURE 1: Cross-linking of subunits *i*, *6*, and *f* with DSP. Mitochondria were isolated from the wild-type strain, and 0.375% Triton X-100 protein mitochondrial extracts were prepared. The two preparations were incubated with the indicated concentrations of DSP for 30 min at 27 °C. The reaction was stopped upon addition of Tris, and samples (30 $\mu$g of protein) were analyzed by Western blot. The blots were incubated with polyclonal antibodies raised against subunits *i*, *f*, and 6. Key: mit, mitochondria; ext, 0.375% Triton X-100 extract.

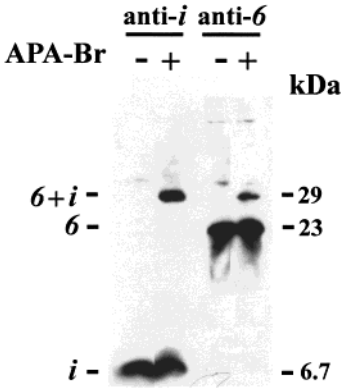


FIGURE 2: Cross-linking involving Cys[23] of subunit *6* and subunit *i*. The 0.375% Triton X-100 extracts were prepared from wild-type mitochondria and incubated with 200 $\mu$M APA-Br. Samples (30 $\mu$g of protein) were submitted to Western blot analyses. The blots were incubated with the indicated polyclonal antibodies.

3-(*N*-maleimidylpropionyl)biocytin, whereas Cys[3] of subunit *i*K3C was accessible only when the membranous barrier was disrupted (not shown), a fact which is very consistent with a $N_{in}-C_{out}$ orientation. This result is in full agreement with that recently reported by Arnold et al. (*28*), who showed by proteolytic cleavage analysis that the C-terminal part of subunit *i* is located in the intermembrane space.

*DSP Induces Cross-Linked Products Involving ATP Synthase Subunits i, f, and 6*. We have previously shown that when a detergent concentration as low as 0.375% Triton X-100 is used, the protein extracts obtained from isolated mitochondria contain a functional ATP synthase that is sensitive to $F_o$ inhibitors (*24*). Preliminary experiments to identify neighboring proteins of subunit *i* were performed with the homobifunctional reagent DSP, which reacts with amino groups distant at a maximum of 12 Å. Wild-type mitochondria and crude Triton X-100 extracts were incubated with increasing concentrations of DSP. Subunit *i* contains six lysine residues, each of them constituting a potential target to cross-linking reagents such as DSP, so it could therefore provide data concerning their environment. Figure 1 shows that two main cross-linked products (having apparent molecular masses of 17 and 29 kDa) were obtained with mitochondria and with Triton X-100 extracts. Identification of cross-linked products was performed by Western blot analysis with the aid of our set of specific antibodies. This demonstrated that the species of 17 and 29 kDa were cross-linked products of $i + f$ and $i + 6$, respectively. It should be noted that the hydrophobic subunit *6*, whose molecular mass is 27 943 Da, displayed an apparent molecular mass of 23 kDa in our electrophoretic conditions. Although mitochondria and Triton X-100 extracts gave qualitatively similar results, incubation of Triton X-100 extracts always gave more intense bands (Figure 1).

*Cys[23] of Subunit 6 Is Involved in the Cross-Links between Subunits 6 and i*. In addition to the identification of the proteins involved in adduct formation with subunit *i*, interesting data concerning the positions from which the cross-links originated were provided by using a unique target in subunit *i*. For this purpose, mitochondria isolated from K3C and K54C strains and Triton X-100 protein extracts were used, their unique cysteine residue being the target of a specific thiol reagent. However, the existence of a unique cysteine residue in subunit *6* (Cys[23]) located near the surface of the outer side of the inner mitochondrial membrane (*23*) could lead to misinterpretations of the data. To clarify this point, a 0.375% wild-type Triton X-100 extract was incubated with the cross-linking reagent APA-Br. At one of its ends this reagent bears a bromide group specific of sulfhydryls and at its other end an azido group. A cross-linked product of 29 kDa involving subunits *6* and *i* was evidenced by Western blot analysis (Figure 2), thus showing that Cys[23] of subunit *6* is located near subunit *i* (at a distance of 9 Å or less). These data imply that the two mutants K3C and K54C would very likely also form these adducts in the presence of a cross-linking reagent specific of thiol groups.

*Disulfide Cross-Link Formation between Cys-Substituted Mutants of Subunit i and Subunits 6 and g*. The presence of a unique cysteine residue not only in subunit *6* but also in the engineered mutants of subunit *i* made feasible the formation of disulfide bridges between the native and engineered subunits of the $F_o$ sector. Disulfide cross-link formation was induced by 1.5 mM Cu(II)−(1,10-phenanthroline)$_3$ (1.5 mM $CuCl_2$ was also used as in ref *19* but similar cross-linking results were obtained with both of these reagents). The 0.375% Triton X-100 protein extracts of the two mutants K3C and K54C were incubated with CuP and analyzed by Western blot (Figure 3). Oxidation of the K3C mutant protein extract led to five bands revealed by anti-subunit *i*, whereas only three were observed with the K54C extracts (Figure 3A). Among the cross-linked products, one with an apparent molecular mass of 29 kDa was identified as the result of the disulfide bridge formation between subunits *6*C23 and *i*K54C. An identical product was obtained with the K29C mutant whose mutation is located at the end of the membrane-spanning segment and for which a strong *i*K29C + *6* adduct was obtained upon CuP-catalyzed oxidation (Figure 3A). This favors the proximity of Cys[54] and Cys[29] of mutant subunits *i* to Cys[23] of subunit *6*. All of these data are consistent with the intermembrane location of residue Cys[23] of subunit *6* and of residues 29 and 54 of subunit *i*. The K54C extracts also displayed a CuP-catalyzed oxidation product of 12 kDa involving subunit *i*K54C and an unidentified small component (Figure 3A). Figure 3A also

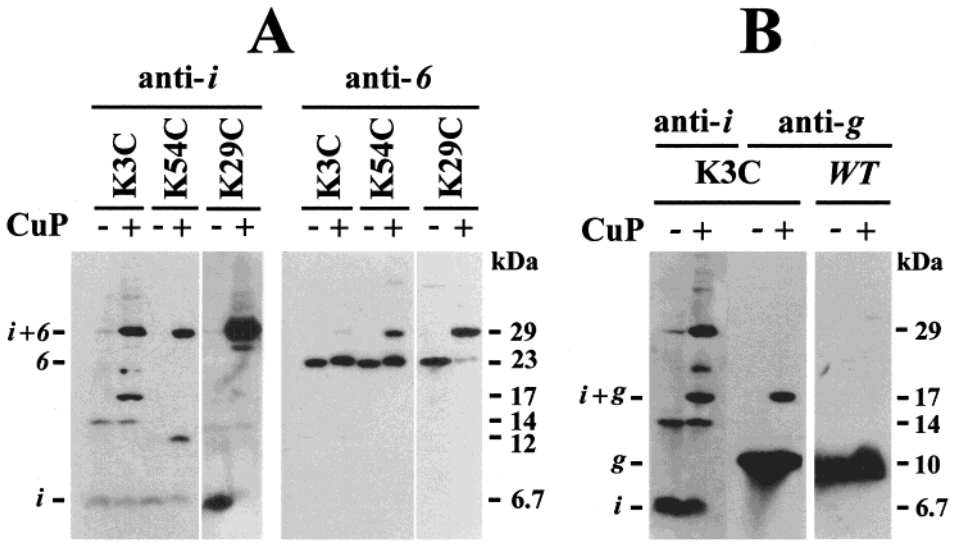


FIGURE 3: Disulfide bridges between components of the yeast $F_o$. The 0.375% Triton X-100 extracts were prepared from mitochondria isolated from K3C, K54C, and K29C strains (A) and from K3C and wild-type (WT) strains (B) and incubated in the presence or in the absence of 1.5 mM Cu(II)−(1,10-phenanthroline)$_3$ (CuP). Samples (30 $\mu$g of protein) were analyzed by Western blot. The blots were incubated with the indicated polyclonal antibodies.

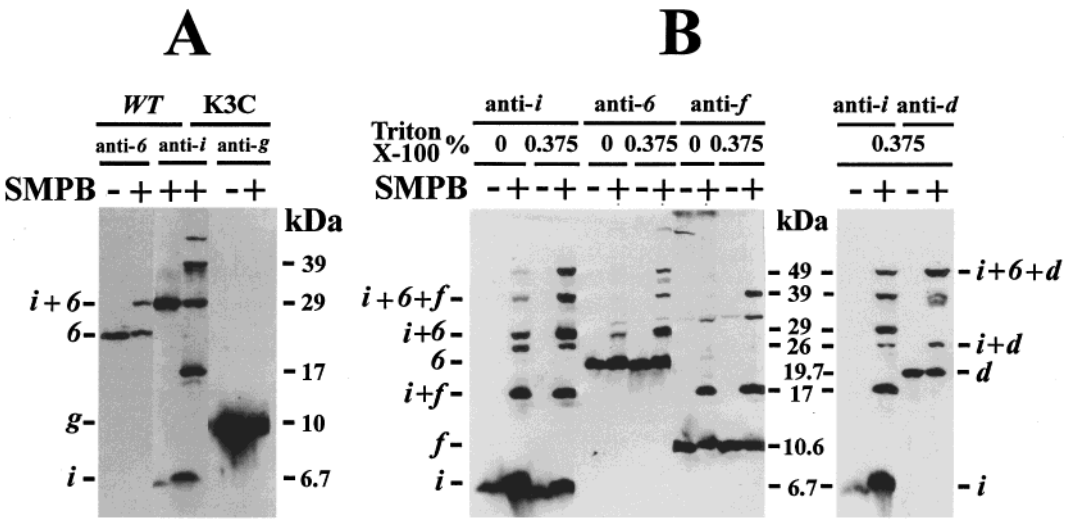


FIGURE 4: Cross-linking of wild-type and K3C mitochondria and Triton X-100 extracts with SMPB. Mitochondria isolated from wild-type (WT) and K3C strains and their 0.375% Triton X-100 extracts were incubated with 200 $\mu$M SMPB. Samples (30 $\mu$g of protein) were analyzed by Western blot. The blots were incubated with the indicated polyclonal antibodies. Panels: (A) wild-type and K3C extracts; (B) K3C mitochondria (0) and K3C 0.375% Triton X-100 extract (0.375).

shows that subunit *i*K3C is involved in the formation of a cross-linked product whose apparent molecular mass is around 29 kDa and which does not contain subunit *6*. The protein involved in this adduct formation with subunit *i* is not a component of the yeast ATP synthase and has not yet been identified. The product of apparent molecular mass 17 kDa formed with the K3C extract was the result of a disulfide bridge formation between subunit *i*K3C and the unique cysteine residue ($Cys^{75}$) of subunit *g*, since the wild-type extract did not display such a cross-link (Figure 3B). Moreover, the K3C extracts showed another band of apparent molecular mass 14 kDa; this product was presumed to be formed by oxidation (Figure 3B).

*Subunit i Is Close to Subunit d, a Component of the Second Stalk*. In another set of experiments, SMPB, which has a spacing arm of 14.5 Å, was used to produce cross-links between thiol and amino groups. As expected, incubation of wild-type mitochondrial Triton X-100 extracts with SMPB led to a cross-linked product of 29 kDa which involves $Cys^{23}$ of subunit *6* and an amino group of the wild-type subunit *i* (Figure 4A). Mitochondrial membranes of the K3C mutant had cross-linked products similar to those of the Triton X-100 extract (Figure 4B). This indicates that membrane extraction by 0.375% Triton X-100 does not significantly modify the relationships between $F_o$ subunits. Upon incubation with SMPB, the K3C extract displayed additional adducts involving subunit *i* but not subunit *g* (Figure 4A), despite the proximity of the two proteins evidenced by a disulfide bond formation (see above). From the additional bands, cross-linked products originating from *i*K3C + *d* and *i*K3C + *6* + *d* were identified. Subunit *d* is a main component of the second stalk located on the matrix side and close to subunit *4* (subunit *b*) (*16*, *40*). Cross-linked products corresponding to *i*K3C + *f* and *i*K3C + *6* + *f* adducts were also identified (Figure 4B). Upon incubation with SMPB, the K54C mitochondrial membranes mainly gave a 41 kDa band

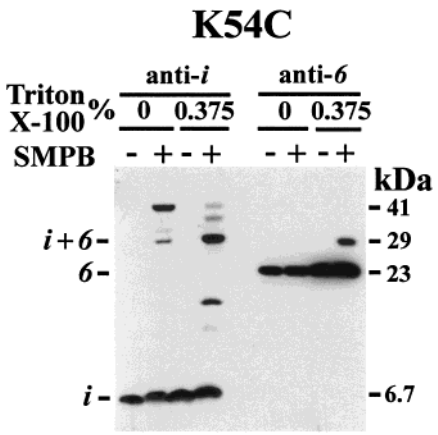


FIGURE 5: Cross-linking of K54C mitochondria and Triton X-100 extract with SMPB. Mitochondria isolated from K54C mitochondria (0) and the K54C 0.375% Triton X-100 extract (0.375) were incubated with 200 $\mu$M SMPB. Samples (30 $\mu$g of protein) were analyzed by Western blot. The blots were incubated with the indicated polyclonal antibodies.

involving *i*K54C and a nonidentified component which is not an ATP synthase subunit. The intensity of the 41 kDa band was largely decreased in the Triton X-100 extract, which displayed one main product made of $i + 6$ (Figure 5).

*Subunit i Forms Cross-Linked Products with Proteins That Are Not Involved in the Structure of the Yeast ATP Synthase.* It appears from the above results that subunit *i* is close to subunits *f*, *6*, *g*, and *d*, four well-characterized components of the ATP synthase complex. In addition, oxidation experiments revealed that three unidentified proteins formed adducts of apparent molecular masses 29 and 14 kDa with *i*K3C and 12 kDa with *i*K54C. Figure 6 shows that the unidentified proteins are not firmly bound to the yeast ATP synthase, since the increase in Triton X-100 concentration highly decreased the intensity of the adducts obtained by oxidation, while the 29 kDa product originating from the disulfide bond formation between $Cys^{23}$ of subunit *6* and $Cys^{54}$ of subunit *i*K54C was still formed in comparable amount upon oxidation of 2% Triton X-100 extracts by CuP or diamide. Similarly, the $i + g$ adduct was no longer formed in a 1% Triton X-100 extract, conditions which dissociate the dimer-specific subunit *g* from $F_o$ (*26*). It has previously been shown that inactivation of the ATP20 gene encoding subunit *g* does not significantly alter the oxidative phosphorylations, thus indicating that subunit *g* is not essential (*41*). All of these results strongly suggest that the above unidentified components, like subunit *g*, are proteins which are not involved in the structure of the yeast ATP synthase.

## DISCUSSION

Subunit *i* is a newly identified component of the yeast ATP synthase (*27*, *28*). This component probably exists in other organisms since open reading frames probably encoding this protein have been identified in *Schizosaccharomyces pombe* (EMBL accession number Z99753), *Neurospora crassa* (GenBank accession number AI329387), and *Caenorhabditis elegans* (GenBank accession number AF067943.1 22834−22974). Whereas Arnold et al. (*28*) reported that deletion of the ATP18 gene led to a strain completely deficient in oligomycin-sensitive ATPase activity and unable to grow on nonfermentable carbon sources, we showed in a recent paper that the mutant strain devoid of subunit *i* could grow on nonfermentable carbon sources such as lactate or glycerol, but more slowly than the wild-type strain, and that mutant mitochondria displayed significant alteration of oxidative phosphorylations (*27*). So far, the discrepancy concerning the phenotype of the null mutant in the ATP18 gene has not been solved, the only difference being the haploid strains used (W303-1A for Arnold and collaborators and D273-10B/A/H/U in our study).

In the present paper, a topological study of subunit *i* was undertaken to define its location in the complex and its possible relationships with other known components of the enzyme. A $N_{in}-C_{out}$ orientation of subunit *i* means that there is a short hydrophilic segment (amino acid residues 1−6) in the matrix and a C-terminal hydrophilic segment containing amino acid residues 29−59 in the intermembrane space.

*Subunit i Forms Cross-Linked Products with ATP Synthase Subunit 6 on the Outside of the Inner Mitochondrial Membrane*. The experiments reported in this paper were carried out first with the homobifunctional reagent DSP. They gave two main cross-linked products involving wild-type subunit *i* and subunits *6* and *f* (these two components being essential subunits of $F_o$). To define more accurately the targets involved and the distances between these subunits, we used the above-mentioned cysteine mutants and various cross-linking procedures. Figure 7 summarizes the data. Subunit *i*, the N-terminus of subunit *6* containing the unique cysteine residue ($Cys^{23}$), and the first membrane-spanning segment of subunit *6* are shown. The different cross-links produced by APA-Br, by SMPB or following sulfhydryl oxidation, are reported. The disulfide bond formation between *6*C23 and *4*D54C was described in a previous paper (*23*). The $6 + i$ cross-linked product obtained with SMPB in a wild-type context involves $Cys^{23}$ of subunit *6* and amino groups of the four lysine residues of subunit *i* present in the intermembrane space. In addition, disulfide bond formation following sulfhydryl oxidation easily occurred between *i*K54C and *6*C23 and mainly between *i*K29C and *6*C23. These data are in favor of an interaction between the first transmembrane α-helix of subunit *6* and the unique membrane-spanning segment of subunit *i*. As the stoichiometry of subunit *6* is 1 per yeast ATP synthase complex (*24*), the quasi-absence of both free subunits *i* and *6* after oxidation of the *i*K29C Triton X-100 extract leads to the conclusion that subunits *i* and *6* display the same stoichiometry of 1.

*Subunit i Forms Cross-Linked Products with ATP Synthase Subunits f, d, and g on the Inner Side of the Inner Mitochondrial Membrane*. Position 3 of subunit *i* is located on the matrix side probably close to the surface of the inner mitochondrial membrane. From this position, cross-linked products were obtained with subunits *f* and *d* with SMPB. We have already reported that subunit *f* has a unique membrane-spanning segment (residues 67−85) and displays a $N_{in}-C_{out}$ orientation (*25*). As a consequence, the product $i + f$ involves a cross-link between $Cys^3$ of the mutant subunit *i* and one of the amino groups from the eight lysine residues of subunit *f* located on the matrix side and at the most at a distance of 14.5 Å. The cross-linking between subunit *i*K3C and subunit *d*, a component of the second stalk of the ATP synthase, shows that a lysine residue of the latter subunit is at the most at a distance of 14.5 Å from *i*K3C

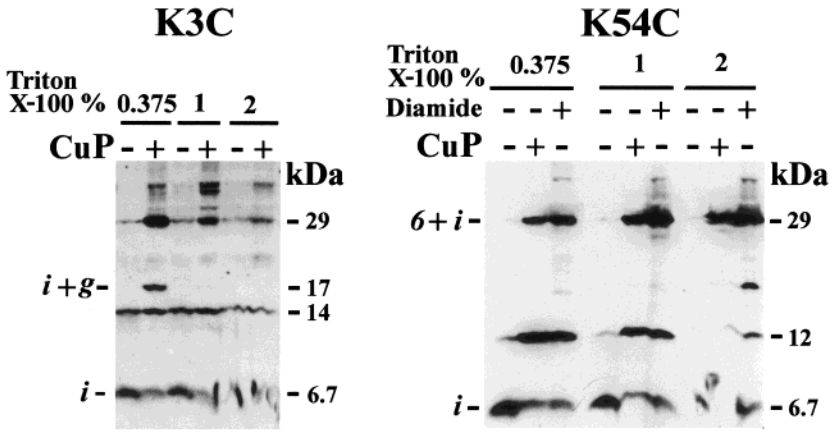


FIGURE 6: Decrease in cross-linked product formation following sulfhydryl oxidation in the presence of high Triton X-100 concentrations. The 0.375%, 1%, and 2% Triton X-100 extracts of mitochondria isolated from K3C and K54C strains were incubated with either 1.5 mM Cu(II)−(1,10-phenanthroline)$_3$ (CuP) or 2 mM diamide as described in Experimental Procedures. Samples (30 $\mu$g of proteins) were analyzed by Western blot. The blots were incubated with polyclonal antibodies raised against subunit *i*.

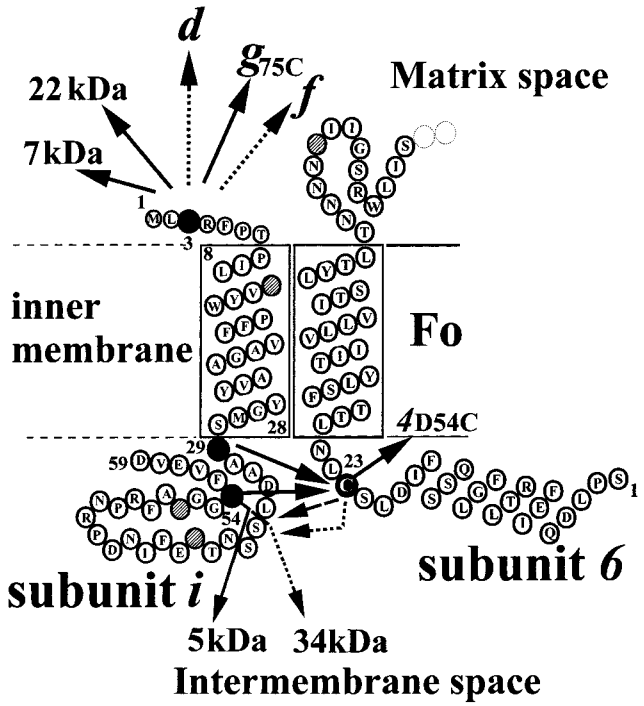


FIGURE 7: Schematic representation showing the near neighbors of subunit *i*. The numbering of subunit *6* begins at serine 1 of the mature sequence (*43*). The first membrane-spanning segment of subunit *6* and part of its first loop located in the matrix space are shown. The black filled circles represent the lysine residues replaced by cysteines in subunit *i*. Other lysine residues are shown as hatched circles. The arrows are oriented toward the various subunits cross-linked by sulfhydryl oxidation (solid arrow), SMPB (dotted arrow), and APA-Br (dashed arrow). *4*D54C stands for subunit *4* modified at position 54 (*23*).

and is therefore close to the inner surface of the inner mitochondrial membrane. With the reagent SMPB, hetero-trimers were obtained. The $i + 6 + f$ adduct involves a first cross-link between *6*C23 and an amino group of the C-terminal part of subunit *i* and a second one between *i*K3C and an amino group of the N-terminal part of subunit *f*. A similar network of cross-links can be proposed for the $i + 6 + d$ product.

The disulfide bond formation between subunit *i*K3C and subunit *g*75C revealed the proximity of the two subunits. Subunit *g* has been described as a dimer-specific subunit associated with $F_o$ (*26*), which is also required for maximum levels of respiration, ATP synthesis, and cytochrome *c* oxidase activity in yeast (*41*). Although its molecular mass is 12 921 Da, it migrates with an apparent molecular mass of 10 kDa on SDS gel electrophoresis. Subunit *g* has a unique membrane-spanning segment (residues 85−105) with a $N_{in}$−$C_{out}$ orientation (*26*). Such an orientation is consistent with the disulfide bridge formation between the Cys$^3$ of the mutant subunit *i* and Cys$^{75}$ of subunit *g* in the matrix space. An intriguing result was the absence of the cross-linked product *i*K3C + *g* in the presence of either SMPB or DSP. As subunit *g* has 10 lysine residues in its matrix part (residues 1−84), this means that these amino acid residues are not accessible to succinimidyl groups of cross-linking reagents. We postulate that a steric hindrance due to either subunit *f* or another component located in this neighborhood prevents cross-linking by SMPB and DSP.

*Subunit i Is Located in the Periphery of the Yeast ATP Synthase*. By CuP-catalyzed oxidation of K3C and K54C Triton X-100 extracts, we have shown the presence of three unknown proteins loosely bound to the yeast ATP synthase and close to subunit *i*. By using our set of polyclonal antibodies we were not able to identify these proteins: This indicates that these components are not subunits of the yeast ATP synthase. This in turn implies that subunit *i* has a peripheral location in the yeast ATP synthase complex, thus allowing contacts with other components of the inner mitochondrial membrane. The question of the specificity of the disulfide bond formation arises from these results. The following points address this issue: (i) From residue 3, three proteins were cross-linked by oxidation, one of them being subunit *g* whose Cys$^{75}$ is also located on the matrix side, while the other two are not components of the yeast ATP synthase. However, on the matrix side, subunits $\alpha$, $\delta$, and $\gamma$ and OSCP have endogenous cysteine residues that have been shown to be accessible to the maleimide reagent *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (*23*) and therefore should be able to react with *i*K3C by oxidation, but in fact they do not. (ii) The unidentified proteins giving the cross-linked products of 14 and 29 kDa with *i*K3C were also obtained in the absence of CuP, thus showing the close proximity of the cross-linked partners. (iii) On the inter-membrane side, the ATP synthase subunits *6*, *9* (*23*), and *e*

(*42*) have a unique cysteine residue but only subunit *6* was cross-linked by oxidation with *i*K54C. (iv) There was a decrease in the amounts of cross-linked products generated by oxidation when Triton X-100 concentrations were increased. This probably means that the unidentified proteins loosely associated with the ATP synthase were removed by the detergent treatment. The numerous cross-links obtained by sulfhydryl oxidation from residues 3 and 54 and not from residue 29 are consistent with a greater susceptibility to thermal collision, perhaps because residues 3 and 54 lie at the N- and C-termini of the protein, respectively. From the apparent molecular masses of the products obtained by CuP-catalyzed oxidation, masses of 22, 7, and 5 kDa can be evaluated for the partners of subunit *i* involved in the disulfide bridge formations. Similarly, the mass of the unidentified partner of subunit *i* in the 41 kDa product obtained by incubation of K54C mitochondrial membranes with SMPB can be evaluated to be 34 kDa. To identify these peripheral proteins, we are now developing experimental procedures which make it possible to keep these proteins associated to the ATP synthase.

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