The Second Stalk of the Yeast ATP Synthase Complex: Identification of Subunits Showing Cross-Links with Known Positions of Subunit *4* (Subunit *b*)†

Vincent Soubannier,‡ Filippo Rusconi,§ Jacques Vaillier,‡ Geneviève Arselin,‡ Stéphane Chaignepain,‡ Pierre-Vincent Graves,‡ Jean-Marie Schmitter,§ Jie Lai Zhang,|| David Mueller,|| and Jean Velours*,‡

Institut de Biochimie et Génétique Cellulaires du CNRS, Université Victor Segalen, Bordeaux 2, 1 rue Camille Saint-Saëns 33077 Bordeaux Cedex, France, Institut Européen de Chimie et Biologie − Ecole Polytechnique, Avenue Pey Berland, BP 108-33402 Talence cedex, France, and Department of Biochemistry and Molecular Biology, The Chicago Medical School, 3333 Greenbay Rd. North Chicago, IL, 60064

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ABSTRACT: A component of the stator of the yeast ATP synthase (subunit *4* or *b*) showed many cross-linked products with the homobifunctional reagent dithiobis[succinimidyl propionate], which reacts with the amino group of lysine residues. The positions in subunit *4* that were involved in the cross-linkings were determined by using cysteine-generated mutants constructed by site-directed mutagenesis of ATP4. Cross-linking experiments with the heterobifunctional reagent *p*-azidophenacyl bromide, which has a spacer arm of 9 Å, were performed with mitochondria and crude Triton X-100 extracts containing the solubilized enzyme. Substitution of lysine residues by cysteine residues in the hydrophilic C-terminal part of subunit *4* allowed cross-links with subunit *h* from C98 and with subunit *d* from C141, C143, and C151. OSCP was cross-linked from C174 and C209. A cross-linked product, *4*+*β*, was also obtained from C174. It is concluded that the C-terminus of subunit *4* is distant from the membrane surface and close to F_1 and OSCP. The N-terminal part of subunit *4* is close to subunit *g*, as demonstrated by the identification of a cross-linked product involving subunit *g* and the cysteine residues 7 or 14 of subunit *4*.

INTRODUCTION

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), base-piece (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 pathway. The connecting stalks are constituted of components from both F_1 and F_o. When the two sectors are coupled, the enzyme functions as a reversible H^+-transporting ATPase or ATP synthase (*1*, *2*). The model for energy coupling by F_oF_1 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 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 that is made of subunits *γ* and *ϵ* and that constitutes 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 and F_o. They form the stator, a second stalk that 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*, *11*). The *E. coli* ATP synthase and the bovine enzyme contain 8 and 16 different types of subunit, respectively (*12*). In the case of *Saccharomyces cerevisiae*, the ATP synthase is composed of at least 13 different kinds of subunit involved in the structure of the enzyme (*13*). Like in the mammalian enzyme, five subunits (*α*, *β*, *γ*, *δ*, and *ϵ*) compose the yeast F_1. The F_o part contains eight different subunits. Three of them are mitochondrially encoded (subunits *6*, *8*, and *9*). Subunits *6* and *9* are homologous to the *a*- and *c*-subunits of *E. coli*, respectively, and subunit *8* is homologous to the A6L subunit of the mammalian ATP synthase. The other five are subunit *4*, which is homologous to the *b*-subunit, OSCP, subunit *d*, subunit *h*, and subunit *f*. In addition, 7 small polypeptides have been identified: IF_1, 9 kDa, 15 kDa, subunits *e*, *g*, *i*, and *k*, which are associated proteins whose disruption of the structural gene does not lead to a lack of assembly of the complex (*13*, *14*).

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* To whom correspondence should be addressed. Tel: 33 5 56999048. Fax: 33 5 56999059. E-mail: jean.velours@ibgc.u-bordeaux2.fr.

‡ Université Victor Segalen.

§ Institut Européen de Chimie et Biologie.

|| The Chicago Medical School.

[1] Abbreviations: APA-Br, *p*-azidophenacyl bromide; ASIB, 1-[*p*-azidosalicylamido]-4-[iodoacetamido]butane; DSP, dithiobis[succinimidyl propionate]; EDTA, ethylenediamine tetraacetic acid; 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; Ni-NTA, nickel-nitrilotriacetic acid; OSCP, oligomycin-sensitivity-conferring protein; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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We have 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. The yeast mitochondrial subunit *4* is a stator component that is homologous to the *b*-subunit of beef heart mitochondrial ATP synthase (*15*). It is made of 209 amino acid residues. Subunit *4* has two domains: the N-terminal part, which is predominantly hydrophobic and in contact with the hydrophobic subunits of the membrane sector, and the C-terminal part, which is charged and hydrophilic and in contact with OSCP (*13*) and subunits *d* and F6 in the bovine enzyme (*16*). Subunit *4* is considered to span across the membrane twice, with the N- and C-terminal parts emerging from the membrane on the F_1 side (*16*). Like its counterpart subunit *b* in bacteria, the mitochondrial *b*-subunit (subunit *4*) appears necessary for a tight coupling between proton flux and ATP synthesis (*17*−*22*). It has been suggested that the *E. coli b*-subunit does not have a rigid rodlike structure but shows an inherent flexibility compatible with a dynamic role in coupling (*23*−*25*). As a consequence, knowledge of the structure of subunit *4* and of its environment is essential to understand the mechanism of F_o and of the whole complex. Other components of the stator are subunits *6* and *f*. Subunit *6* is mitochondrially encoded (*26*) and is involved in the proton channel, like its counterpart the *E. coli a*-subunit. It is an intrinsic protein that is likely to be composed of 5 transmembrane α-helices (*27*). The N-terminal part of the yeast subunit *6* is located in the intermembrane space (*28*). Subunit *f* is another integral inner membrane protein that spans across the membrane once (amino acid residues 67 to 85) with a N_{in}-C_{out} orientation (*29*, *30*), and which is close to subunit *4* (*28*, *30*). In this report, we provide new data concerning the stator of the yeast enzyme and particularly the structural elements of subunit *4* most probably interacting with subunits *d*, *g*, *h*, OSCP, and *β*.

EXPERIMENTAL PROCEDURES

Materials. ASIB and DSP were from Pierce. APA-Br, bradykinin, α-cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, neurotensin, and adrenocorticotropic hormone were from Sigma. Oligonucleotides were purchased from Genset Corporation. Endoproteinase Lys-C was from Boehringer Mannheim. 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 (*31*). 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 *4* were obtained after complementation of the deleted-disrupted yeast strain PVY10 (MATα, *met6*, *ura3*, *his3*, ATP4::URA3) by the low copy shuttle vector pDR1 (*32*) containing the wild-type ATP4 gene and the mutated versions of ATP4 gene, respectively. The K7C-$(His)_6$ mutant was constructed according to the following strategy. A *Aat*II restriction site was introduced into the ATP4 gene by site-directed mutagenesis of the phagemid pDR1ATP4 with the oligonucleotide 5′-TCTAAATTGAAGACGTCACAACAG-3′. This removed the stop codon of the ATP4 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 pDR1ATP4 phagemid. This resulted in a subunit *4* having the additional sequence TSHHHHHH in its C-terminus. A 1220 bp *Xho*I DNA fragment encoding the C-terminal part of subunit *4*K7C was removed from the pDR1ATP4 phagemid and replaced by the *Xho*I DNA fragment encoding the C-terminal part of the subunit *4*-$(His)_6$. The resulting construct was used to complement the PVY10 strain.

The gene for subunit *g* of the yeast ATP synthase (ATP20) was cloned by two sequential PCRs. The first PCR used total genomic DNA isolated from strain D273-10B/A/H/U. The PCR product from this reaction was amplified with primers having unique restriction sites, *Eco*R1 and *Hind*III, that were used in the next cloning step. The PCR products were digested with *Eco*RI and *Hind*III and ligated into the plasmid pRS306, thus resulting in pATP20-6. The creation of the null mutant in ATP20 was done by a PCR-based mutagenesis (*33*). Yeast D273-10 B/A/H/U was transformed with the PCR product. Transformants (ΔATP20) were selected and checked by PCR to ensure the correct integration event.

Biochemical Procedures. Cells were grown aerobically at 28 °C in a complete liquid medium containing 2% lactate as carbon source (*34*) and harvested in logarithmic growth phase. Mitochondria were prepared as described previously (*35*) 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., (*36*) in the presence of 5% SDS with bovine serum albumin as the standard protein. Specific ATPase activity was measured at pH 8.4 according to Somlo (*37*). Crude mitochondrial extracts and cross-linking experiments with APA-Br and ASIB were performed as described in ref *28*.

Cross-Linking 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%, 2%, or 4% of 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 200 *μ*M DSP for 30 min at 27 °C. The reaction was quenched by the addition of 10 mM Tris. In other experiments, ATP synthase isolated from wild-type yeast was purified as in ref *38*, but the enzyme was purified by ion exchange chromatography and eluted with a buffer containing 0.25 M sucrose, 0.1% Triton X-100, 90 mM NaCl, and 22 mM triethanolamine-HCl, pH 8.0. Twenty five micrograms of the enzyme (0.5 mg/mL) was incubated with 5 *μ*M DSP.

Purification of a 4+g Cross-Linked Product. Mitochondrial membranes (50 mg protein in 10 mL of the isolation buffer adjusted at pH 7.4) of the K7C-$(His)_6$ mutant were incubated with 0.5 mM APA-Br as described in ref *28*. Purification of $(His)_6$-tagged proteins was done as in ref *39*. The proteins were eluted from the beads by addition of 330 *μ*L of a buffer containing 20 mM EDTA, 0.1% SDS, and 50 mM Tris-maleate, pH 6.0. The collected eluates were lyophilized. SDS was removed from the dried material by addition of 1.6 mL of acetone/acetic acid/triethylamine/water

(85:5:5:5) (*40*). The dried pellet was dissolved in 100 μL of 0.1% SDS, 5 mM Tris-maleate, pH 6.8, and dialyzed against 500 mL of the previous buffer for 12 h at room temperature. Finally, the sample was lyophilized, submitted to SDS-gel electrophoresis, and stained with Amido Black. The selected bands were cut and destained, and the proteins they contained were cleaved in situ at 37 °C with endoproteinase Lys-C for 16 h (*41*). The resulting peptides were extracted and dried (*42*).

Mass Spectrometry Analysis. Mass spectrometry analyses were performed on a REFLEX III MALDI-TOF mass spectrometer from Bruker (Bremen) in the reflector mode (accelerating voltage, 20 kV; reflector voltage, 22.8 kV; delayed extraction time, 250 ns). The amount of peptide analyzed was around 1 pmol. Calibration was performed externally unless otherwise specified. Bradykinin, neurotensin, and adrenocorticotropic hormone were used for quadratic calibration of the mass spectrometer. α-Cyano-4-hydroxycinnamic acid and 3,5-dimethoxy-4-hydroxycinnamic acid were used as matrixes. The peptidic mixtures were analyzed after residual acrylamide gel removal and concentration in a micro chromatography device as described previously (*42*).

Cross-Linked Protein Identification Procedure. Identification of the unknown cross-linked protein by mass spectrometry was performed as a two-step procedure. In the first step, the peptidic mixture resulting from the proteolysis of the 24 kDa band was analyzed by MALDI-TOF mass spectrometry. The most intense mass peaks that could be unambiguously assigned to theoretically expected subunit *4*K7C-$(His)_6$ peptides were used as internal markers for the second step. In this second step, analysis of the peptidic mixture resulting from the proteolysis of the 36 kDa band provided mass peaks that could not be assigned to subunit *4*K7C-$(His)_6$. These unassigned masses were compared, using the ProFound utility (located at http://prowl.rockefeller.edu/cgi-bin/ProFound), to those of the *S. cerevisiae* peptide database calculated from NCBI's nr protein database. This yielded a list of candidate proteins ranked according to their calculated posterior probability (*43*). To definitely confirm the identity of the protein that was cross-linked with subunit *4*K7C-$(His)_6$, we processed one peptide whose intense mass peak could not be assigned to subunit *4*K7C-$(His)_6$ further by post-source decay analysis. The fragment ions of this peptide were then compared with the MS-Tag utility from the UCSF Mass Spectrometry Facility (Baker, P. R., and Clauser, K. R. http://prospector.ucsf.edu) to the *S. cerevisiae* fragment database calculated from NCBI's nr protein database.

SDS−Polyacrylamide Gel Electrophoresis and Western Blot Analyses. SDS gel electrophoresis was done according to Laemmli (*44*) with 15% polyacrylamide slab gels. The slab gels were stained with Amido Black or silver-stained according to Ansorge (*45*). Western blot analyses were described previously (*38*).

RESULTS

DSP Induces Many Cross-Linked Products Involving Subunit 4. Preliminary experiments to identify neighboring proteins of subunit *4* were performed with the homobifunctional reagent DSP, which has a spacer arm of 12 Å. Crude Triton X-100 extracts of wild-type mitochondria were incubated with DSP. We have previously shown that at low detergent concentrations such as 0.375% Triton X-100, the extracts contain a functional ATP synthase that is sensitive to F_o inhibitors (*46*). Subunit *4* contains 20 lysine residues. These residues constitute potential targets to cross-linking reagents and thereby provide data concerning their local environment. Identification of cross-linked products was performed by Western blot (Figure 1). Seven different bands in the molecular mass range of 23−70 kDa reacted with our antibody raised against subunit *4*. Two of these bands with apparent molecular masses of 36 and 38 kDa were absent from cross-linked products obtained with the mitochondrial extracts at Triton X-100 concentrations of 1% or 2%. Purified F_oF_1 ATP synthase displayed only two major cross-linked products. This indicates that some neighboring proteins were displaced or removed from the complex by high Triton X-100 concentrations or by the ATPase purification procedure as shown earlier (*47*). The absence of the 36 and 38 kDa products correlated with the appearance of a new product having a molecular mass of 33 kDa (Figure 1). This 33 kDa band was attributed to a *4*+*f* adduct (not shown); this cross-linked product was also present after reaction of DSP with the purified F_oF_1 ATP synthase.

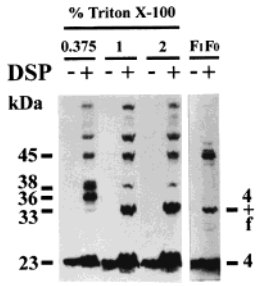

FIGURE 1: Western blot analysis of crude mitochondrial Triton X-100 extracts and ATP synthase cross-linked by DSP. Mitochondrial Triton X-100 extracts (0.375%, 1%, 2%) and purified ATP synthase isolated from wild-type yeast were cross-linked with 200 and 5 μM of DSP, respectively, as described in the Experimental procedures. Samples (30 μg of mitochondrial protein and 7.5 μg of ATP synthase) were dissociated and analyzed by Western blot. The blots were probed with anti-*4* polyclonal antibodies at a dilution of 1:10000. The designations *4* and *4*+*f* are for subunit *4* and for the cross-linked product involving subunits *4* and *f*, respectively.

Cross-Linkings from Subunit 4 and Involving OSCP, Subunits h, d, and the β-Subunit. In addition to the identification of protein partners cross-linked with subunit *4*, interesting data concerning the positions involved in the cross-links were provided by using unique targets in subunit *4*. Since subunit *4* has 20 lysine residues, we undertook an analysis of the 20 positions by constructing 20 different mutants, each having a single cysteine residue replacing the lysine residue of interest. In this case, cross-linking experiments were performed with the heterobifunctional reagent APA-Br, which has a spacer arm of 9 Å. The mutated versions of ATP4 gene were borne by the low copy vector, pDR1. All mutants grew with glycerol or lactate as carbon source with doubling times ranging from 220 to 270 min. The specific ATPase activities of isolated mitochondria and sensitivities to F_o inhibitors were not significantly altered (not shown), thus indicating that oxidative phosphorylation was not significantly altered by the cysteine substitutions.

Mitochondria and mitochondrial Triton X-100 extracts isolated from the 20 strains were incubated with APA-Br or

Table 1: Apparent Molecular Masses of Cross-Linked Products Induced by APA-Br and Involving Subunit *4*[a]

strains	apparent molecular masses of cross-linked products involving subunit *4*		
	membrane	0.375% Triton X-100 (kDa)	1% Triton X-100 (kDa)
wild type (PVY193)	–	–	–
K7C	36	36	–
K7C−$(His)_6$	36	-	–
K12C	-	36^{tr}	–
K14C	36	36	–
K30C	-	-	–
K71C	-	-	–
K78C	45^{tr}	45^{tr}	45
K86C	–	–	–
K87C	–	–	–
K98C	43	43	43
K104C	–	–	–
K121C	–	–	–
K128C	–	–	–
K141C	45	45	45
K143C	45^{tr}	45	45
K151C	45	45, 70	45
K174C	45^{tr}, 70^{tr}	45, 70	45, 70
K188C	–	–	–
K192C	–	–	–
K207C	45^{tr}	45^{tr}	45^{tr}
K209C	45	45	45

[a] Mitochondria, 0.375% and 1% (w/v) Triton X-100 extracts were incubated with APA-Br, as described in the Experimental Procedures. Western blot analyses were done, and blots were reacted with anti-*4* polyclonal antibodies. The PVY193 strain was the PVY10 strain complemented with the low copy vector bearing the wild-type ATP4 gene. Superscript "tr" = traces.

ASIB and analyzed by Western blot. The apparent molecular masses of APA-Br-induced cross-linked products are shown in Table 1. Except for K7C and K14C mutants, the mitochondrial membranes and the Triton X-100 protein extracts of each mutant showed similar patterns of cross-linked products. Identical results were obtained with ASIB (not shown). Protein identification was determined by Western blot analyses of APA-Br-induced cross-linked mitochondrial Triton X-100 extracts using our set of specific antibodies. Further studies on K78C and K207C mutants are not reported here because the intensities of the 43 and 45 kDa bands, respectively, were very low and did not permit identification of the cross-linked proteins. Unambiguous results corresponding to six positions in subunit *4* are reported in Figures 2 and 3. APA-Br induced cross-linking depended on the position of the cysteine residue in subunit *4*. Subunit *4*K98C allowed cross-link to subunit *h* upon reaction with APA-Br (Figure 2A). Although the molecular mass of subunit *h* is 9953 Da, the *4*+*h* adduct has an apparent molecular mass of 43 000. We have already reported an anomalous migration of subunit *h* in our electrophoretic conditions: this hydrophilic and acidic subunit showed a apparent molecular mass of 15 kDa (*38*). With Cys at positions 141, 143, and 151, the cross-linking was mainly with subunit *d* (Figure 2B). The *d*+*4*K143C cross-linked products showed 3 bands close to each other. This was interpreted as cross-linkings involving different positions of subunit *d*, thus leading to products showing slightly different mobilities. Moreover, even in the absence of APA-Br, there was a 45 kDa product that was cross-reactive with the anti-subunit *d* antibody. The intensity of this band was dependent on the sample and was always lower than the APA-Br induced cross-linked products. For Cys at positions 174 and 209 of subunit *4*, the cross-linking was with OSCP (Figure 3A). The nearly identical molecular masses of OSCP and subunit *d* led to adducts with subunit *4* that had the same apparent mass of 45 kDa (Figures 2 and 3). Moreover, for

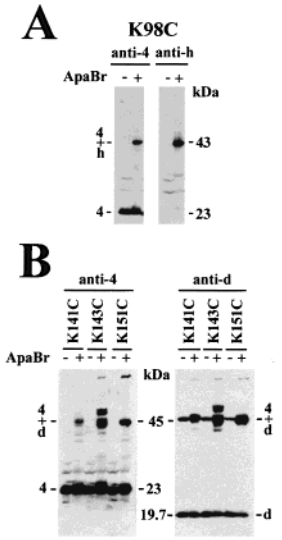

FIGURE 2: Cross-linking of subunits *4* and *h* and subunits *4* and *d*. Triton X-100 (0.375%) extracts of mitochondria isolated from yeast mutants K98C, K141C, K143C, and K151C were incubated with 200 μM APA-Br as in ref *28*. After irradiation at 365 nm for 10 min, samples (30 μg of protein) were dissociated and analyzed by Western blot: (A) blots were probed with anti-*4* (dilution 1:10000) and anti-*h* (dilution 1:7500) antibodies; (B) blots were probed with anti-*4* (dilution 1:10000) and anti-*d* (dilution 1: 20000) antibodies. The designations *4*,*d* and *4*+*h*,*4*+*d* are for subunit *4*,*d* and for the cross-linked products involving subunits *4*, *h*, and *d*, respectively.

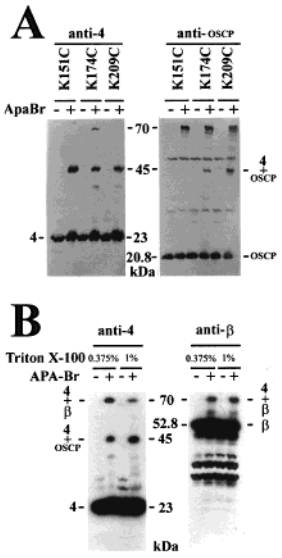

FIGURE 3: Cross-linking of subunit *4* with OSCP, subunits *4* and β. Triton X-100 (0.375%) extracts of mitochondria isolated from yeast mutants K151C, K174C, and K209C were incubated with 200 μM APA-Br and analyzed by Western blot as in Figure 2: (A) blots were probed with anti-*4* (dilution 1:10000) and anti-OSCP (dilution 1:10000) polyclonal antibodies; (B) 0.375% and 1% Triton X-100 extracts of K174C mitochondria were cross-linked with APA-Br and analyzed as in Figure 2. The blots were probed with anti-*4* (dilution 1:10000) and anti-β (dilution 1:30000) polyclonal antibodies. The designations *4*, OSCP, *4*+OSCP, and *4*+β are for subunit *4*, OSCP and for the cross-linked products involving subunits *4*, OSCP and β, respectively.

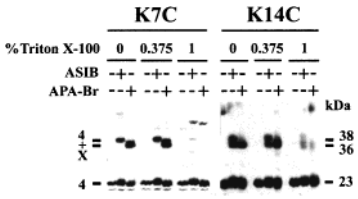

FIGURE 4: Cross-linking of subunit *4* from C7 and C14 with a protein loosely bound to the ATP synthase. Mitochondria and 0.375% and 1% Triton X-100 extracts of mitochondria isolated from yeast mutant K7C and K14C were incubated with 200 μM APA-Br or 200 μM ASIB as in Figure 2. Samples (30 μg protein) were submitted to a Western blot analysis. Blots were reacted with anti-*4* (dilution 1:10000) polyclonal antibodies. The designation *4* is for subunit *4*.

the three mutants tested, the anti-OSCP antibody revealed cross-linked products in the molecular mass range of 70 kDa. These products have not been identified so far, but they could originate from the reaction with the endogenous C100 of OSCP with another yet unidentified protein (Figure 3A). Some unidentified faint bands that were present without treatment with APA-Br gave a cross-reaction with anti OSCP antibodies. For Cys 174, a cross-link with the β-subunit was detected (Figure 3B). This product was obtained either with mitochondrial membranes (Table 1) or with the 0.375% and 1% Triton X-100 mitochondrial extracts containing the solubilized enzyme. In Figure 3B, the film was overexposed to detect the cross-linked product and resulted in the appearance of nonspecific bands and of proteolytic products of the β-subunit.

Cross-Linking of Subunits 4 and g. From positions 7 and 14 of subunit *4*, cross-linking with ASIB and APA-Br resulted in protein adducts of 38 and 36 kDa (Figure 4). These two bands were absent in the cross-linked products obtained with 1% Triton X-100 protein extracts, suggesting that these neighboring proteins are loosely associated to the ATP synthase. As subunit *4* has a molecular mass of 23 kDa, the associated protein(s) should display a molecular mass of around 13−15 kDa. These bands did not react with any of the antibodies tested except for the anti-subunit *4*. Purification of the APA-Br cross-linked product was performed according to the following strategy. A $(His)_6$-tag was placed at the C-terminus of subunit *4*K7C. The addition of the $(His)_6$ tag did not alter either the cell growth with lactate or glycerol as carbon source or the sensitivity of ATPase toward inhibitors (not shown). However, when compared with the K7C mutant, the enzyme was poorly extracted using Triton X-100. This might explain why the 0.375% Triton X-100 extract of K7C-$(His)_6$ mutant mitochondria was unable to produce the APA-Br cross-linked product of 36 kDa (not shown). However, this adduct was obtained with K7C-$(His)_6$ mitochondrial membranes. As expected, addition of the $(His)_6$ tag at the C-terminus of subunit *4* led to a small increase in the molecular mass of subunit *4* (Figure 5A, lane 2). Subunit *4*K7C-$(His)_6$ and the adduct were purified by Ni-NTA chromatography followed by SDS-gel electrophoresis (Figure 5A, lane 3). The identification of the cross-linked product involving subunit *4* was confirmed by Western blot (Figure 5B).

To obtain a sufficient amount of cross-linked product for qualitative analysis, we incubated 50 mg of mitochondrial protein with APA-Br. The sample was separated by SDS gel electrophoresis, the gel was stained with Amido Black,

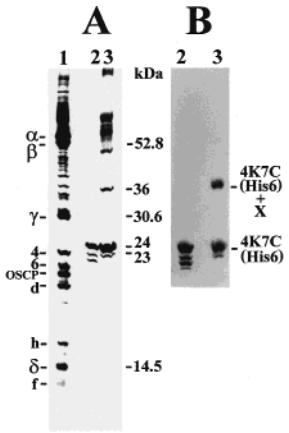

FIGURE 5: Purification of the cross-linked product involving C7 of subunit *4*. Mitochondria isolated from the yeast strain K7C-$(His)_6$ were incubated or not with 0.5 mM APA-Br as in Figure 2. The purification strategy is described in the Experimental Procedures. Samples were dissociated and subjected to SDS-gel electrophoresis: (A) silver-staining of the slab gel; (B) the slab gel was transferred and the blot was probed with anti-*4* (dilution 1:10000) polyclonal antibodies. Lane 1, purified wild-type ATP synthase (7.5 μg); lane 2, (control) subunit *4*K7C-$(His)_6$ extracted from 5 mg of mitochondrial protein and purified by Ni-NTA chromatography (one-sixth of the sample was analyzed); lane 3, products extracted from 5 mg of K7C-$(His)_6$ mitochondrial protein cross-linked by APA-Br and purified by Ni-NTA chromatography (one-half of the sample was analyzed). The designation *4*K7C-$(His)_6$ is for subunit *4*K7C having a $(His)_6$-tag at the C-terminus.

Table 2: Mass Spectrometric Analysis of Endoproteinase-LysC Peptides of Subunit *4*K7C-$(His)_6$ and *4*K7C-$(His)_6$+g Adduct

		$[M + H]^+$ *m/z*	
position	sequence	calculated	measured
subunit *4*			
[88−98]	VSDVLNASRNK	1202.649	1202.650
[175−188]	SVISRVQSELGNPK	1513.834	1513.820
[129−141]	ETVELESEAFELK	1523.747	1523.760
[15−30]	ANSIINAIPGNNILTK	1652.933	1652.950
[193−207]	VLQQS...QLLSK	1714.959	1714.930
[105−121]	DRIDS... AETTK	1903.972	1903.960
[193−209]	VLQQ...QLLSKLK	1956.138	1956.150
[152−174]	AVLDS...RQLAK	2759.496	2759.470
subunit *g*			
[41−52]	EGLQPPTVAQFK	1314.705	1314.700
[61−77]	QSLNF.....VLSCLK	1891.036	1891.040
[87−108]	YGAYG...EIIGRRK	2460.340	2460.290[a]
[87−113]	YGAYG...KLVGKY	3020.672	3020.380

[a] Hypothetical subunit *g* peptide selected for post-source decay analysis in order to confirm that the cross-linked protein is bona fide subunit *g*.

the pieces of gel containing the 24 kDa and 36 kDa bands were cut, and the proteins they contained were cleaved by endoproteinase Lys-C. The resulting peptides were extracted and analyzed by MALDI-TOF mass spectrometry (see Experimental procedures). Table 2 shows the masses of peptides obtained by endoproteolytic cleavage of the isolated 24 and 36 kDa bands. Eight of the peptide fragments of the cross-linked product digest were attributed to subunit *4*K7C-$(His)_6$. By comparing the masses of unidentified peptides with the *S. cerevisiae* peptide database, we determined that there was only one protein with a calculated probability of 1.0: subunit *g* (Table 2). Subunit *g* was thus considered to be the best candidate in the formation of the adduct with subunit *4*. However, the formal identification of the cross-

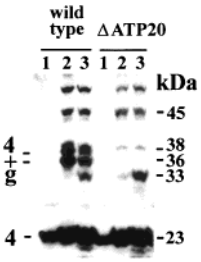

FIGURE 6: Western blot analysis of Triton X-100 extracts of mitochondria isolated from wild type and ΔATP20 yeast and cross-linked by DSP. Samples were prepared and incubated with 200 μM DSP as in Figure 1. Lanes 1, mitochondria without DSP (control); lanes 2, 0.375% Triton X-100 extract; lanes 3, 1% Triton X-100 extract. The blot was probed with anti-*4* (dilution 1:10000) polyclonal antibodies. The designations *4* and *4*+*g* are for subunit *4* and for the cross-linked product involving subunits *4* and *g*, respectively.

linked protein was performed in a post-source decay analysis experiment, by analyzing fragmentations originating from the parent ion of $[M+H]^+$ monoisotopic *m*/*z* 2460.29 likely to correspond to the segment [87−108] of the subunit *g* whose calculated $[M+H]^+$ monoisotopic *m*/*z* is 2460.34. The *m*/*z* values of the largest fragment peaks were analyzed (as described in Experimental Procedures), and a single *S. cerevisiae* protein was found to generate a fragmentation pattern similar to that observed, that is, subunit *g* of the ATP synthase. Indeed, 19 intense mass peaks corresponding to fragments of the parent peptide were selected and analyzed with the post-source decay analysis utility. Among them, only three did not match the fragmentation pattern of the subunit *g*, whereas the perfectly matching masses corresponded to the following fragment series: a5-a8, b5-b8, y6-y9. Finally, the immonium ions corresponded perfectly to the peptide composition. This identification was thus considered to be definitive.

Subunit *g* appears to behave as a loosely associated protomer of the ATP syntheses complex. It has been shown to be involved in the dimerization of the yeast ATP synthase (*14*). The gene encoding subunit *g* (ATP20) was deleted in the wild-type strain D273-10B/A/H/U. The resulting strain ΔATP20 could grow on media containing nonfermentary carbon sources, showing that this protein is not essential for oxidative phosphorylation. However, like most of the null mutants in ATP synthase genes (*48*), rho- cells accumulated during growth on media containing fermentary carbon sources (not shown). The ATP synthase complex isolated from ΔATP20 mitochondria was found to be assembled and functional. Incubation of Triton X-100 mitochondrial protein extracts of the ΔATP20 strain with DSP was performed, and the samples were analyzed as before. Figure 6 clearly shows that the 36 and 38 kDa bands were absent from the cross-linked products in full agreement with the data described above in Figure 5 and Table 2.

DISCUSSION

In this paper, we report data on the relationships between subunit *4* and other components of the stator of the yeast ATP synthase. Belogrudov et al. (*49*) have already reported that disuccinimidyl tartarate cross-links the bovine subunit *b* with OSCP, subunits *d* and F6, and the β-subunit. Cross-linking of wild-type yeast mitochondrial Triton X-100

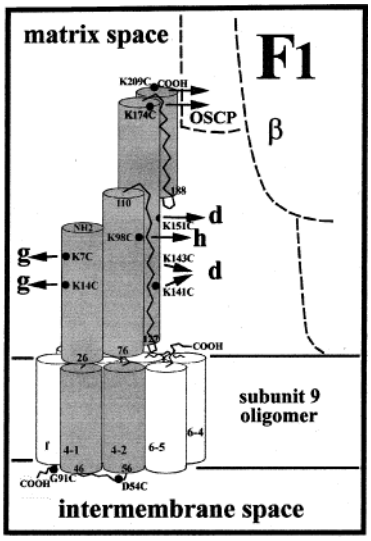

FIGURE 7: Proposed secondary and tertiary structures of subunit *4* and its environment in the complex. The cylinders represent α-helices, the gray one belonging to subunit *4*. The black circles are cysteine residues that were introduced by site-directed mutagenesis in previous work (*28*, *30*) and in this work. K209 is the C-terminal residue of subunit *4*.

extracts by the homobifunctional reagent DSP also resulted in many bands involving the amino group of lysine residues of subunit *4*. To refine the analysis of these results, we performed our study with a series of mutated subunits *4*, each of them bearing a unique cysteine to target a hetero-bifunctional cross-linking reagent, such as APA-Br or ASIB. Among the twenty different mutants analyzed by Western blot, only eight of them gave intense cross-linked products involving subunit *4* with 5 other subunits of the yeast ATP synthase that were unambiguously identified.

Cross-Linkings Involving the C-Terminal Part of Subunit 4. A scheme showing the topology of subunit *4* and its neighboring proteins constituting the hydrophobic part of the ATP synthase is proposed in Figure 7. Subunit *4* is shown with two transmembrane segments linked by a short hydrophilic loop (amino acids 46−56) whose amino acids 54 and 55 protrude in the intermembrane space (*28*), the rest of the molecule being directed toward the matrix space (*50*). Subunit *6* is shown with five membrane-spanning segments on the basis of prediction methods (*27*) and of topological results reported on the homologous *E. coli a*-subunit (*39*, *51*). The putative α-helix *4-2* of subunit *4* is shown in contact with the α-helices *6-4* and *6-5* of subunit *6*: this is based on the proton dissipating pathways occurring in subunit *4*L68R, V69E mutant (*22*). The unique hydrophobic α-helix of subunit *f* is assumed to be in interaction and in contact with the membrane-spanning α-helix *6-1* and far from the aspartate residue 54 of subunit *4*, an assumption based on cross-link experiments done with ASIB and APA-Br with a D54C mutant (*28*, *30*).

The hydrophilic C-terminal part of subunit *4* (residues 76−209) has been predicted to be outside of the lipid bilayer and mostly α-helical (*52*). If such a continuous α-helix were to exist, 80 amino acid residues would be sufficient to traverse the distance between the top of F_1 and the lipid bilayer surface (*53*). Thus, these 133 residues are likely folded. It has been reported that trypsin could cleave the bovine *b*-subunit first between Arg166 and Gln167 and then

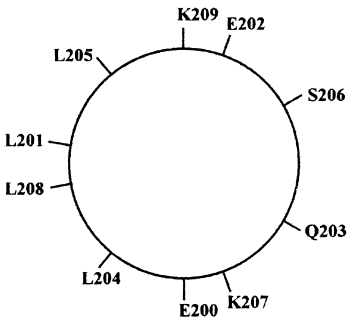

FIGURE 8: Positions of residues 200−209 of the yeast subunit *4* in a putative α-helical wheel.

at the consecutive positions, Arg120-Arg121 and Arg121-His122 (*50*, *54*). These results are consistent with the idea that these amino acids are located in loops connecting α-helices. We propose a model of subunit *4* in which the C-terminal part is organized in three α-helical segments connected by large loops. In the bovine enzyme, the C-terminal part of the molecule is involved in interactions with other components of the stator. The region stretching from the amino acid residues 121−166 is the main binding site of subunit *b* for subunit *d* (*16*). In our model, subunit *h* interacts with the 76−110 α-helical segment of subunit *4*. Subunit *d* was cross-linked from the 127−174 α-helical segment, which is in agreement with the results of Collinson et al. (*16*). An interesting result is the cross-linking of OSCP and the *β*-subunit from position 174 of subunit *4* and only OSCP from position 209 (the C-terminal residue). These data are in agreement with previous results showing an in vitro interaction between the C-terminal segment made of the last 133 amino acid residues of subunit *4* and OSCP (*13*). Modeling of the last 10 residues of subunit *4* as an α-helix reveals an amphipathic structure (Figure 8). The C-terminus of the *E. coli b*-subunit displays a very similar structure (*55*) with a hydrophobic face probably interacting with the *δ*-subunit, the protein homologous to OSCP. Although subunit *4* is unique in the mitochondrial complex (*16*, *46*, *56*), it is generally considered to be homologous to the *E. coli b*-subunit, which is a dimer in the bacterial complex. The C-terminal domains of this dimer interacts with the *β*-subunit (*53*, *55*). In addition, the C-terminus of the *E. coli b*-subunit was cross-linked with an α-subunit at αC90. As a result, it has been proposed that the *b*-subunit extends from the membrane surface to the N-terminal domain of the α-subunit (*53*). On the basis of the homology between OSCP and the *E.coli δ*-subunit, we conclude that the C-terminal part of subunit *4* is located far from the membrane surface and close to the *β*-subunit, and that the hydrophobic face of the α-helix composed of the last 10 amino acid residues is a domain of interaction with OSCP.

Cross-Linkings Involving the N-Terminal Part of Subunit 4. The N-terminal part of subunit *4* is close to subunit *g*, since it was cross-linked from positions C7 and C14, but not from C12. From these data, we suggest that the N-terminal part of subunit *4* is a α-helical segment that interacts with the hydrophilic part of subunit *g*, an integral inner-membrane protein (*14*, *29*). The yeast subunit *g* contains 115 amino acid residues corresponding to a molecular mass of 12921 Da. It has a unique transmembrane α-helix (from residues 84 to 105). A N_{in}-C_{out} orientation has been proposed for the homologous bovine protein (*29*). In our study, subunit *g* peptides having masses of 2460.3 and 3020.6 were identified by mass spectrometry analysis. These two peptides contain the postulated hydrophobic domain and the C-terminal part of subunit *g*. Since they were not modified by the cross-linking experiment, the hydrophilic N-terminal part of subunit *g*, which is directed toward the matrix space like the N-terminus of subunit *4*, is probably the target of the azido group of APA-Br.

A few small supernumerary subunits are loosely associated to the yeast enzyme, such as subunits *e* and *g* (*14*, *47*). The absence of the 36 kDa and 38 kDa bands in the cross-linked products of 1% and 2% Triton X-100 extracts and of the purified enzyme (Figure 1) probably reflects the removal of subunit *g* during the extraction. With K7C mitochondria and 0.375% Triton X-100 extract, the heterobifunctional reagents APA-Br and ASIB having spacer arms of 9 and 18.8 Å led to protein adducts of 36 and 38 kDa, respectively, whereas the homobifunctional reagent DSP (with a spacer arm of 12 Å) gave these same two bands with the wild-type 0.375% Triton X-100 extract. Moreover, K14C mitochondria and the 0.375% Triton X-100 extract showed the same two bands in the presence of ASIB (Figure 4). Indirect evidence that the 38 kDa adduct involves subunit *g* was provided by the absence of the 36 and 38 kDa species when incubating mitochondrial Triton X-100 extracts of ΔATP20 strain with DSP. Therefore, we argue that both the 36 and 38 kDa bands are cross-linked products involving subunits *4* and *g* and that their difference in electrophoretic mobilities reflects either cross-linkings involving different positions of subunit *g* or different conformations of the cross-linking reagents that led to different access to subunit *g*.

DSP did not lead to the *4*+*g* adduct with 1% Triton X-100 extracts of wild-type mitochondria but instead gave a cross-linked product of subunits *4* and *f*. Subunit *g* might impede the *4*+*f* adduct formation in the 0.375% Triton X-100 extract by steric hindrance. We have previously reported a cross-linking between *4* and *f* from the position 54C of subunit *4* by using ASIB (*28*, *30*). This linkage occurred on the outer face of the inner mitochondrial membrane. As all the lysine residues of subunit *4* and the largest part of subunit *f* (residues 1−66), which contains 8 of the 9 lysine residues, are located in the matrix space, the formation of a *4*+*f* cross-linked product in the presence of DSP is conceivable (Figure 1). However, cross-linking of the 20 different 1% Triton X-100 mutant extracts obtained with either APA-Br or ASIB did not make it possible to identify which Lys residue(s) of subunit *4* was (were) involved in the linkage between subunits *4* and *f* by DSP. By using heterobifunctional reagents reacting with thiol groups at one end and with the amino group of lysine residues at the other end, we have experiments in progress to elucidate this point.

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