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The *Saccharomyces cerevisiae* ATP Synthase[1]

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The ATP synthase of the yeast *Saccharomyces cerevisiae* is composed of 20 different subunits whose primary structure is known. The organization of proteins that constitute the membranous domain is now under investigation. Cysteine insertions combined with the use of nonpermeant maleimide reagents and cross-linking reagents showing different lengths and specificity contribute to the knowledge of the location of the N- and C-termini of the subunits involved in the stator of the enzyme and their organization. This review summarizes data on yeast ATP synthase obtained in our laboratory since 1980.

KEY WORDS: Yeast; ATP synthase; subunits; F_0 organization; crosslinking.

INTRODUCTION

ATP synthesis is a fundamental biochemical process mainly present in mitochondria, chloroplasts, and bacteria. This reaction, which occurs on an membranous heterooligomeric enzyme, the ATP synthase, uses an electrochemical transmembrane proton gradient generated by an electron flux along a redox proteic chain. The numerous studies done with this enzyme have recently led to the rotative molecular motor concept. This enzyme, which has a molecular mass of nearly 600 kDa, is composed of two domains. The hydrophilic domain F_1 bears three catalytic sites and the hydrophobic domain F_0 contains a proton channel. The model for energy coupling by ATP synthase that has gained the most general support is the binding change mechanism (Boyer, 1993). 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 (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998). The catalytic sites are alternatively modified, thus leading to ATP formation from ADP and phosphate during the rotation of the central component γ inside the $\alpha_3\beta_3$ hexamer (Noji *et al.*, 1997). Proton translocation through F_0 could promote the rotation of an oligomer of hydrophobic components (Sambongi *et al.*, 1999; Panke *et al.*, 2000), leading to the rotation of the ε and γ subunits. As a result, the prokaryotic enzyme (the simplest enzyme) appears to consist of a rotor composed of subunits γ, ε, and c_{9-12} and of a stator containing subunits *a*, b_2, α_3, and β_3. The eukaryotic enzymes have homologous components but, in addition, displays supernumerary subunits involved in the structure and regulation. Thus, the beef enzyme is composed of 16 different subunits (Collinson *et al.*, 1994). In the case of *S. cerevisiae*, the ATP synthase is composed of at least 13 different subunits involved in the structure of the enzyme; the disruption of each of their structural genes leads to a lack of assembly of the ATP synthase (Velours *et al.*, 1998). Seven additional proteins have also been identified that are associated to the yeast enzyme, but are not essential to cell growth with glycerol or lactate as carbon source. Recently, crystallization of the yeast enzyme has revealed the structure at 3.9Å resolution of the main part of F_1 and that of the subunit *9* oligomer of F_0 (Stock *et al.*, 1999). The yeast ATP synthase composition is close to that of mammals and, therefore, can

[1] Key to abbreviations: DCCD, dicyclohexylcarbodiimide; DSP, dithiobis(succinimidyl propionate); F_0 and F_1, integral membrane and peripheral portions of ATP synthase; HPLC, high-performance liquid chromatography; OSCP, oligomycin sensitivity-conferring protein; SDS, sodium dodecyl sulfate.

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be considered as a model. *Saccharomyces cerevisiae* offers the advantage of a facultative aerobic microorganism, which allows the genetic manipulation of genes encoding mitochondrial proteins that are indispensable for aerobic growth, since yeast can obtain its energy from fermentation when oxidative phosphorylations are deficient. Since its doubling time is short, its genome is entirely known, and its genetic manipulation is easy, yeast is as an experimental model for studying the energetic transducing systems, their regulation, and their biogenesis. In this review, structural and functional data of components of the yeast enzyme and topological studies concerning subunits belonging to the F_0 domain are reported.

RESULTS AND DISCUSSION

Purification of the Yeast ATP Synthase

Many procedures have been described to purify the enzyme of *S. cerevisiae* (Tzagoloff and Meagher, 1971; Ryrie, 1975; Roth and Nelson, 1981). We have developed a strategy to obtain large amounts of ATP synthase in order to perform the chemical analysis of its components. The enzyme was extracted from mitochondrial membranes by 0.375% (w/v) Triton X-100. Higher detergent concentrations decrease the oligomycin-sensitive ATPase activity, thus pointing to a disconnection between F_1 and F_0 (Spannagel *et al.*, 1998a). The detergent extract was centrifuged at 100,000 × *g*, the supernatant was diluted twice, and centrifuged at 300,000 × *g* to pellet the enzyme. The enzyme was then purified by molecular sieving chromatography followed by ion-exchange chromatography (Vaillier *et al.*, 1999). By using this procedure, Triton X-100 concentration remains at 0.1% during the purification and loosely bound proteins *e* and *g* remain associated to the ATP synthase. Figure 1 shows SDS-PAGE of the yeast enzyme and Table I provides useful information on its 20 components.

The Subunits γ, δ, and ε of the Catalytic Domain F_1

The catalytic sector F_1 of *S. cerevisiae* is composed of five different subunits that are similar to those of other eukaryotes. The genes encoding the α and β subunits were isolated by Takeda *et al.* (1986) and Saltzgaber-Muller *et al.* (1983), respectively. The subunits γ, δ, and ε were isolated in our laboratory from F_1 purified by conventional procedures (Takeshige *et al.*, 1976). The five components of the catalytic sector were separated in one run by reverse phase HPLC (Arselin *et al.*, 1991).

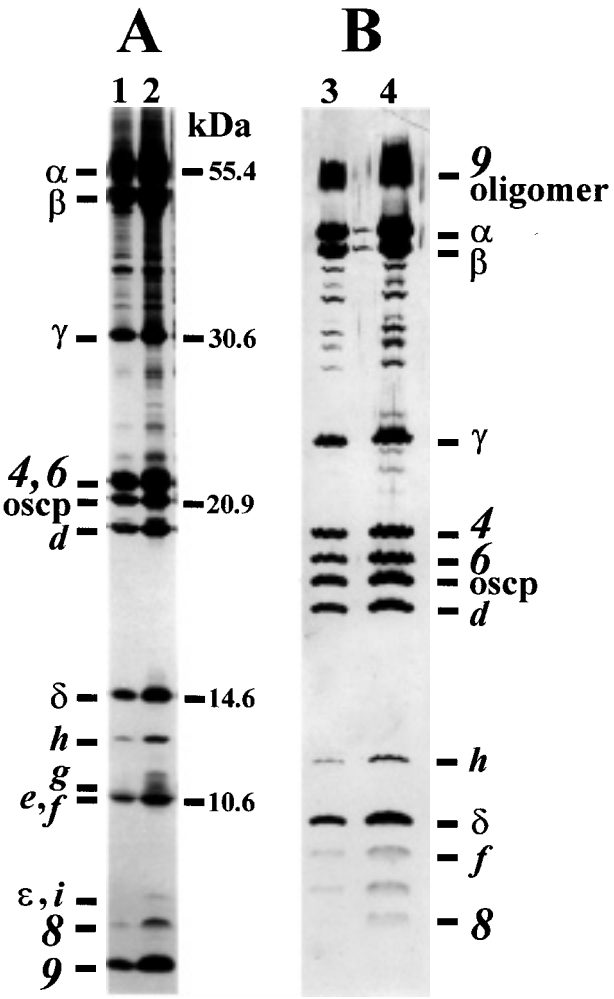

Fig. 1. SDS-polyacrylamide gel electrophoresis of yeast ATP synthase. (A) Tricine SDS-PAGE according to Schägger and Von Jagow (1987); lanes 1 and 2:16 and 32 μg protein, respectively; (B) 15% polyacrylamide slab gel according to Laemmli (1970); lanes 3 and 4: 7 and 15 μg protein, respectively.

The smallest component of F_1 (ε) is 27% identical to that of beef. The yeast ε subunit is composed of 61 amino acid residues. The protein has been entirely sequenced and the ATP15 gene has been isolated, sequenced, and disrupted (Guélin *et al.*, 1993). The null mutant strain is unable to grow on nonfermentable carbon sources. Null mutant mitochondria, prepared from cells grown with galactose as carbon source, display a high respiration rate that is oligomycin-sensi-

Table I. *Saccharomyces cerevisiae* ATP Synthase Subunits[a]

	Subunit	Mammalian nomenclature	Amino acids (mature subunit)	Mass (Da) (mature subunit)	Stoichiometry	p*I* (theoretical)	Gene	Swiss-Prot. accession number
F_1	α	α	510	54,955	3	6.73	*ATP1*	P07251
	β	β	478	51,256	3	5.22	*ATP2*	P00830
	γ	γ	278	30,616	1	9.06	*ATP3*	P38077
	δ	δ	138	14,553	1	4.87	*ATP16*	Q12165
	ε	ε	61	6,611	1	9.77	*ATP15*	P21306
F_0	*6*	*a*	249	27,957	1	7.84	*ATP6* (*OLI2*)	P00854
	8	A6L	48	5,850	1	9.99	*ATP8* (*AAP1*)	P00856
	9	*c*	76	7,787	9–12	7.93	*ATP9* (*OLI1*)	P00841
	4	*b*	209	23,251	1	7.83	*ATP4*	P05626
	OSCP	OSCP	195	20,871	1	9.30	*ATP5*	P09457
	d	*d*	173	19,722	1	8.92	*ATP7*	P30902
	f	*f*	95	10,567	n.d.	9.94	*ATP17*	Q06405
	h	F6	92	10,408	n.d.	4.24	*ATP14*	Q12349
Associated proteins	IF_1	IF_1	63	7,383	1	9.15	*INH1*	P01097
	9 kDa		63	7,286	n.d.	9.00	*STF1*	P01098
	15 kDa		83	9,483	n.d.	7.93	*STF2*	P16965
	e	*e*	95	10,744	n.d.	5.84	*TIM11* (*ATP21*)	P81449
	g	*g*	115	12,921	n.d.	9.82	*ATP20*	Q12233
	i		59	6,687	1	9.70	*ATP18*	P81450
	k		68	7,534	n.d.	8.97	*ATP19*	P81451

[a] n.d., Not determined. The p*I* values of mature subunits do not take into account N-terminal modifications.

tive, corresponding to an uncoupling at F_0 level. Although F_0 components are still present in the null mutant, the F_0 structure is affected by the absence of the ε subunit, leading to a hypersensitivity toward oligomycin. A I_{50} value ten times lower than that half inhibiting either the ADP-stimulated respiration rate or the ATPase activity of the wild-type mitochondria has been reported. In addition, the specific ATPase activity is three times lower than that of wild-type mitochondria and a low stability of F_1 in the absence of ε subunit has been observed (Guélin *et al.*, 1993). In addition, *rho*$^-$ cells arise spontaneously. We observed that plating a few million null mutant cells on nonfermentable carbon sources led to some clones that were able to grow. This spontaneous reversion means that the ε subunit is not essential, in agreement with Lai-Zhang *et al.* (1999). However, purification of F_1 from such a revertant is unsuccessful because of a low stability of the ε subunit-less enzyme (unpublished results).

The yeast δ-subunit is 35% identical to the bovine δ subunit. It is 138 amino acids long with a mass of 14,553 Da. The null mutant is unable to grow on nonfermentable carbon sources. Mutant mitochondria have a residual ATPase activity that is three times lower than that of *rho*$^-$ mitochondria isolated from spontaneous *rho*$^-$ cells and display an unstable F_1 (Giraud and Velours, 1994). This was interpreted as a lack of assembly of F_1 in the absence of δ. The null mutation dramatically affects mitochondrial DNA stability, since inactivation of the ATP16 gene leads to 100% cytoplasmic *petite* (*rho*$^-$ or *rho*°) cells. The null mutant strain displays a slow-growth phenotype on fermentable medium. We have proposed that such a *petite* negative phenotype could be the consequence of a low mitochondrial transmembranous potential $\Delta\Psi$ (Giraud and Velours, 1997). The latter is indispensible for mitochondrial biogenesis (Gasser *et al.*, 1982). The existence of a membrane potential $\Delta\Psi$ in a *rho*$^-$ or *rho*° context is due to the ADP/ATP translocase, which exchanges the cytosolic ATP for the ADP produced by F_1. The slow growth of the null mutant atp16 on fermentable medium has been explained by the lack of F_1 assembly, which decreases the amount of ADP available for the exchange. In contrast, Lai-Zhang *et al.* (1999) suggest that the low growth rate may be due to the inefficiency of the ATPase inhibitor protein to control ATP hydrolysis by F_1 lacking δ or γ subunit. This is not in agreement with the finding that *rho*$^-$ cells appear devoid of F_1 inhibitor (Ebner and Maier, 1977).

The γ subunit is synthesized as a precursor with a long 33 amino acid transit sequence. The null mutant

in ATP3 gene is devoid of a stable F_1 oligomer that is not attached to F_0 and mitochondria have a negligible ATPase activity, although normal amounts of α and β subunits have been detected. A dual structural and functional role of the γ subunit has been reported, one in the catalysis of ATP hydrolysis/synthesis and the second in assembly/stability of F_1 (Paul *et al.*, 1994). As above, cells of the null mutant atp3 are *rho* and display a *petite* negative phenotype (Lai-Zhang *et al.*, 1999).

The Mitochondrially Encoded Subunits *6, 8,* and *9* of the Yeast F_0

The yeast F_0 is composed of three essential hydrophobic subunits (subunits *6, 8,* and *9*) also called proteolipids that are encoded by the mitochondrial genome. These components that are extractable from mitochondria by a mixture of chloroform/methanol (1/1) were separated by reverse-phase HPLC with a gradient of organic solvents (Michon *et al.*, 1988). Subunit *9* (Sierra and Tzagoloff, 1973) is homologous to the *E. coli c* subunit (Fillingame *et al.*, 2000), which is involved in coupling H^+ transport to rotary catalysis during ATP synthesis. It binds DCCD, an inhibitor of ATP synthase (Sebald *et al.*, 1979). The yeast subunit *9* behaves like an oligomer in electrophoresis performed according to Laemmli (1970) (see Fig. 1B), but the latter is unstable in the presence of organic solvents (Velours *et al.*, 1987). Subunit *6* (Murphy *et al.*, 1980; Macino and Tzagoloff, 1980) is homologous to the *a* subunit of *E. coli* and beef. It was predicted as a five membrane-spanning segment protein (Cox *et al.*, 1986). The N-terminus is in the intermembrane space (Michon *et al.*, 1988; Spannagel *et al.*, 1998b) and is posttranslationally cleaved before a SPLDQ sequence also found in subunit *6* of other species (Guélin *et al.*, 1991). At position 176 of the mature protein, subunit *6* contains the conserved Arg210 residue (*E. coli* numbering) that is involved in proton translocation through F_0. Subunit *8* is the third subunit that is translated on the mitochondrial ribosomes (Esparza *et al.*, 1981; Velours *et al.*, 1982; Macreadie *et al.*, 1982). It is a 48-amino acid-long protein (Macreadie *et al.*, 1983; Velours *et al.*, 1984) that is homologous to the A6L product of mammalian mitochondria. Like subunit *9*, this protein begins with *N*-formylMet. A unique membranous spanning segment is predicted for subunit *8*. The unique lysine residue Lys47 is located on the matrix side of the inner mitochondrial membrane (Velours and Guérin, 1986), which gives a N_{out}–C_{in} orientation for the protein. Subunit *8* is not involved in a proton-channel function, but it is required for assembly of subunit *6* and not subunit *9* into ATP synthase (Hadikusumo *et al.*, 1988).

The Nuclear-Encoded Subunits *e, f, h,* and *i* of the Yeast F_0

Subunit *f* has been extracted from the yeast enzyme by 6 M guanidinium chloride and purified by reverse-phase HPLC (Spannagel *et al.*, 1997). Protein- and peptide-sequencing data are in agreement with the sequence of the predicted product of the gene D9481.21 identified on the *S. cerevisiae* chromosome IV. The mature protein displays a molecular mass of 10,567 Da for 95 amino acid residues. The protein is basic with a unique postulated hydrophobic segment (amino acid residues 66 to 84). A N_{in}–C_{out} orientation has been proposed from alkylation experiments of cysteine residues inserted by site-directed mutagenesis (Roudeau *et al.*, 1999). The ATP17 gene encoding subunit *f* is essential since null mutant strains are unable to grow on nonfermentable medium. Null mutant mitochondria still contain an active F_1, but are devoid of F_0 subunits *f, 6,* and *8* while subunits *4,* OSCP, *d,* and *9* are still present.

Subunits *e* and *i* have been extracted from the enzyme and purified as described for subunit *f* (Vaillier *et al.*, 1999). Subunit *e* is loosely associated to ATP synthase, since 1% Triton X-100 removes the protein from the enzyme. Subunit *e* is encoded by the ATP21 genes, which is also called TIM11 gene (Arnold *et al.*, 1977). It migrates as a broad band on SDS–PAGE at the same level as subunit *f* (Fig. 1A). Subunit *e* is involved in the dimerization of the yeast ATP synthase (Arnold *et al.*, 1998).

Subunit *i*, also named *j* (Arnold *et al.*, 1999), is a protein, which probably exists in other organisms, since open reading frames encoding this component have been found in *S. pombe* (EMBL accession number Z99753), *Neurospora crassa* (Gene bank, accession number AI329387) and *Caenorhabditis elegans* (Gene bank accession number AF067943.1 22834..22974). The N-terminal part of subunit *i* has been sequenced, thus allowing the isolation of the structural gene ATP18 (Vaillier *et al.*, 1999). It encodes a protein of 59 residues, showing a unique transmembrane hydrophobic domain (residues 8–28), and having a N_{in}–C_{out} orientation (Arnold *et al.*, 1999; Paumard *et al.*, 2000). The null mutant atp18 shows a slow growth on nonfermen-

table medium and mutant mitochondria display a low ADP/O ratio and a decrease with time in proton pumping upon ATP addition, thus indicating a proton defect through F_0. Like subunit *e*, subunit *i* is not involved in the structure of the yeast enzyme.

Subunit *h* is not apparently related to any subunit described in ATP synthases of higher eukaryotes. Reverse-phase HPLC analysis of an ATP synthase guanidinium hydrochloride extract displayed a hydrophilic component that ran ahead of the ε subunit, the smallest subunit of the F_1. The N-terminal part of the protein was sequenced and the ATP14 gene was isolated. Mature subunit *h* is composed of 92 amino acids for a molecular mass of 10,408 Da and is an acidic protein. It is essential for growth on nonfermentable carbon sources and null mutant mitochondria retain an ATPase activity that is oligomycin-insensitive. In such a mutant, subunit *6*, which is an essential component of the proton channel, and subunit *f* are absent (Arselin *et al.*, 1996; Spannagel *et al.*, 1997). In the absence of subunit *h*, a high percentage of *rho*$^-$ cells arise spontaneously. Research in nucleotide data banks have shown that an open reading frame in *S. pombe* (TrEMBL accession number 059673) and *Botryotinia fuckeliana* (Gene bank accession number AL115386) encodes an homologous protein to subunit *h*.

Subunits *4* and *d*, Two Components of the Second Stalk

Subunit *4*, also called subunit *b*, is composed of 209 amino acid residues. It is 21% identical with the *b* subunit of beef. There is only one copy of subunit *4* per ATP synthase (Spannagel *et al.*, 1998a; Bateson *et al.*, 1999). It is an amphiphilic protein extracted from whole mitochondrial membranes by a mixture of organic solvents (Velours *et al.*, 1987). Purification of the protein has been performed by gel permeation chromatography in the presence of SDS. This protein has two membrane-spanning segments linked by a short hydrophilic loop (residues 46–56) accessible from the intermembrane space (Spannagel *et al.*, 1998b). The N- and C-termini are located on the matrix side. The gene encoding subunit *4* (ATP4 gene) has been isolated (Velours *et al.*, 1988) and disrupted. The null mutant strain atp4 is unable to grow on nonfermentable carbon sources and produces spontaneous *rho*$^-$ cells. Mutant mitochondria isolated from cells grown with galactose still display an ATPase activity, which is oligomycin-insensitive. Analysis of F_0 components showed the absence of subunit *6*, while subunits *8* and *9* were still present (Paul *et al.*, 1989). Analysis of mutants having short versions of subunit *4* demonstrated that the removal of the last 10 amino acid residues led to an oligomycin-insensitive ATPase activity (Paul *et al*., 1992). We interpret this result as the loss of interaction of the C-terminal part of subunit *4* with OSCP, another component of the second stalk (Uh *et al.*, 1990). We have shown a physical interaction between the hydrophilic part of subunit *4* (residues 76–209) and OSCP (1) by comigration of the two proteins during gel permeation chromatography, (2) by the two-hybrid method, and (3) by cross-linking experiments (Velours *et al.*, 1998; Soubannier *et al.*, 1999). So far, no essential amino acid residues in subunit *4* have been found. However, insertion of two hydrophilic and charged amino acid residues in the second membrane-spanning segment (L68R, V69E) led to a strain that is unable to grow at 37°C on nonfermentable medium because of the lack of assembly between F_1 and F_0. At 28°C the mutant strain grows slowly by oxidative phosphorylation. The structure of the mutant F_0 is highly altered since it displays proton-dissipating pathways. In addition, the mutant strain is oligomycin-resistant since the I_{50} value of oligomycin inhibition of ATPase and ATP synthase activities is two- to three-fold higher than that of wild-type mitochondria, thus showing an alteration of the target to oligomycin (Razaka *et al.*, 1994).

Mature subunit *d* is 173 amino acids long. Its NH_2-terminal Ser is blocked by an acetyl group and it is not processed other than by removal of the initiator methionine. The protein is predominantly hydrophilic. It is 22% identical and 44% homologous to the bovine subunit *d*. The null mutant in the *ATP7* gene is unable to grow on nonfermentable carbon sources. Null mutant mitochondria have an active F_1, but the ATPase activity is oligomycin-insensitive, in agreement with the absence of subunit *6* (Norais *et al.*, 1991). The null mutants atp4 and atp7 have a very similar phenotype and integration of the mitochondrially encoded subunit *6* at least depends on the presence of the two nuclear-encoded subunits *4* and *d*. We have proposed that although subunit *6* is synthesized in atp4 null mutant, it is not integrated in the membrane and could disappear upon proteolysis (Paul *et al.*, 1989).

Topological Analysis of the Yeast F_0

The combination of cysteine-generated mutants, nonpermeant maleimide reagents and cross-linking

reagents is now providing new insights into the mitochondrial ATP synthase F_0. Nonpermeant maleimide reagents have been incubated with intact mitochondria having unique engineered targets on various subunits. The C-terminal parts of subunits *f* and *i* were found to be accessible from the intermembrane space (Roudeau *et al.*, 1999; Paumard *et al.*, 2000). Similarly cysteine residues introduced at positions 54 and 55 of the postulated loop of subunit *4* and Cys23 (the unique endogenous cysteine residue of mature subunit *6*) have been found in the intermembrane space (Spannagel *et al.*, 1998b). These data are based either on the protection of the thiol group from alkylation by nonpermeant maleimide reagents or on the modification of the electrophoretic migration rate of the protein upon alkylation by these reagents. Data concerning orientation of subunits *f* and *i* are in full agreement with those reported by Belogrudov *et al.*, (1996) and Arnold *et al.*, (1999), respectively, who used proteolytic cleavage experiments.

Near Neighbors of Subunit *4*

Near-neighbor relationships involving subunit *4* have been investigated by using bifunctional and heterobifunctional reagents having different lengths and specificity. Cross-linking experiments have been done (1) with mitochondrial membranes, (2) with Triton X-100 extracts, and (3) with purified ATP synthase. From Cys54 and Cys55 located in the intermembrane space (see above), subunit *4* was crosslinked with subunit *f*, the distance between the two targets was between 9 Å and 18.8 Å. Azidophenacylbromide (9 Å arm) crosslinked *4*D54C and subunit *6*. Furthermore, a disulfide bridge was also obtained by oxidation between *4*D54C and *6*C23 (Fig. 2). These data are in favor of a close proximity of the first transmembrane-spanning segment of subunit *6* and the second transmembrane-spanning segment of subunit *4* (Spannagel *et al.*, 1998b). In the absence of oxidation, mitochondrial membranes of both mutants *4*D54C and *4*E55C also contain a homodimer of subunit *4* linked by a disulfide bond. As there is only one subunit *4* per ATP synthase, this spontaneous crosslinking has been interpreted as the proximity of ATP synthases in the inner mitochondrial membrane (Spannagel *et al.*, 1998a). The existence of yeast ATP synthase dimers has also been demonstrated by blue native PAGE (Arnold *et a1.*, 1998).

Many cross-linked products involving subunit *4* have been obtained with the bifunctional reagent DSP (a specific reagent of amino groups at a maximum distance of 12 Å). To identify the targets modified by DSP and since subunit *4* contains 20 lysine residues, 20 different mutants, each having a lysine replaced by a cysteine, were constructed. With azidophenacylbromide as a cross-linking agent, mutations in the hydrophilic C-terminal part of subunit *4* allowed crosslinks with subunit *h* from Cys98 and with subunit *d* from Cys141, Cys143, and Cys151. OSCP was crosslinked from Cys174 and Cys209, the C-terminal residue of subunit *4*. A *4* + β cross-linked product was also obtained from Cys174. In the N-terminal part of subunit *4*, cysteine residues at positions 7 or 14 crosslinked subunit *g* (Soubannier *et al.*, 1999), a protein loosely associated with ATP synthase and involved in the dimerization of the enzyme (Arnold *et al.*, 1998).

Near Neighbors of Subunit *i*

Cross-linking experiments performed with DSP on mitochondrial membranes or Triton X-100 extracts have given two products (*i* + *6* and *i* + *f*). Identification of the positions involved in these crosslinks have been obtained from cysteine-generated mutants. Mutants *i*K3C, *i*K29C, and *i*K54C have been constructed (Fig. 2). Positions 3 and 54 are located in the matrix space and in the intermembrane space, respectively (Arnold *et al.*, 1999; Paumard *et al.*, 2000). On the matrix side, but close to the membrane, crosslinks involving the Cys3 of subunit *i* and amino groups of subunits *d* and *f* are obtained with the heterobifunctional reagents succinimidyl-4-(*p*-maleimidophenyl)-butyrate and *N*-(α-maleimidoacetoxy)succinimide ester, which have spacer arms of 11.6 Å and 4.4 Å, respectively. Upon oxidation, the crosslinked product *i* + *g* was also obtained. The latter adduct is the result of the disulfide bridge formation between *i*K3C and the unique cysteine residue (Cys) of subunit *g*. On the intermembrane space another disulfide bridge was also obtained between position 54 of subunit *i* and Cys23 of subunit *6*. Position 29 is also located on the outer side of the inner mitochondrial membrane, *i*K29C gives a strong adduct with *6*C23 by oxidation, thus showing the proximity of the two cysteines. The absence of both native subunits *i* and *6* upon oxidation, suggests a similar stoichiometry of one per enzyme. These data suggest interactions between the unique membrane-spanning segment of subunit *i* and the first

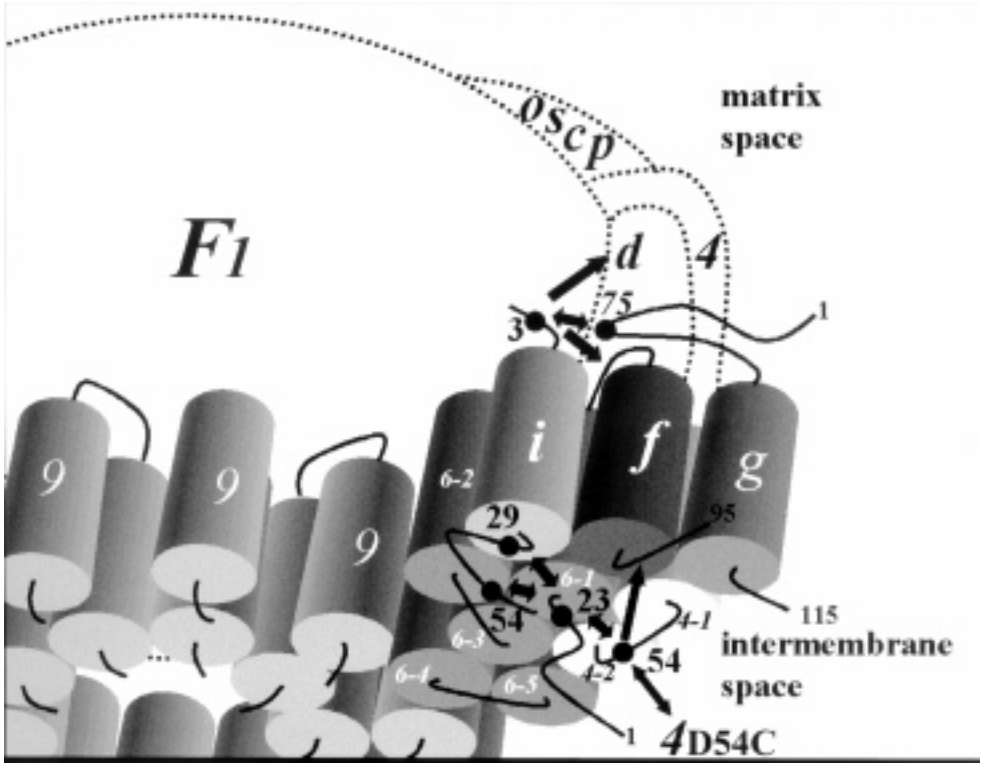

Fig. 2. Schematic representation of the yeast F_0 and targets involved in crosslinking. The cylinders represent the transmembranous α-helixes of subunits *9*, *6*, *4*, *i*, *f*, and *g* observed from the intermembrane space. The numbered black circles are the cysteine residues involved in crosslinking and accessibility experiments; ⇔ crosslink obtained by oxidation; ⇒, crosslink obtained by a heterobifunctional reagent.

transmembranous α-helix of subunit *6* (Paumard *et al.*, 2000).

Zero cross-linked products involving subunit *i* have also been obtained by oxidation from positions 3 and 54 with unknown proteins of 5 kDa, 7 kDa, and 23 kDa that are loosely bound to ATP synthase, thus suggesting 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 enzyme.

CONCLUDING REMARKS

The primary structure of the constituents involved in the structure and the function of yeast ATP synthase is now known. Their organization and finally the complete three-dimensional structure of this object is the next hurdle to cross. This will probably lead us to the detailed understanding of the mechanism and regulation of ATP synthesis. Another point under investigation is the identification of the mitochondrial components in the close environment of the eukaryotic ATP synthase.

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