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Subunit f of the Yeast Mitochondrial ATP Synthase: Topological and Functional Studies

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Modified versions of subunit f were produced by mutagenesis of the ATP17 gene of Saccharomyces cerevisiae. A version of subunit f devoid of the last 28 amino acid residues including the unique transmembranous domain complemented the oxidative phosphorylation of the null mutant. However, a two-fold decrease in the specific ATP synthase activity was measured and attributed to a decrease in the stability of the mutant ATP synthase complex as shown by the low oligomycin-sensitive ATPase activity at alkaline pH. The modification or not by non-permeant maleimide reagents of cysteine residues introduced at the N and C termini of subunit f indicated a N_{in}-C_{out} orientation. From the C terminus of subunit f it was possible to cross-link subunit 4 (also called subunit b), which is another component of the F_0 sector and which also displays a short hydrophilic segment exposed to the intermembrane space.

KEY WORDS: Yeast, mitochondria, ATP synthase, ATP17 gene, subunit f, orientation, cross-linking.

INTRODUCTION

The mitochondrial ATP synthase is the major enzyme responsible for the aerobic synthesis of ATP. The enzyme resolves into two parts. The catalytic sector F_1, with subunits α, β, γ, δ and ε, which is a water-soluble unit retaining the ability to hydrolyze ATP when in a soluble form, and a detergent soluble unit, the F_0 sector which is embedded in the membrane and is composed of hydrophobic subunits forming a specific proton pathway. In addition a connecting stalk is composed of components of both F_1 and F_0. When the two sectors are coupled, the enzyme functions as a reversible H^+-transporting ATPase or ATP synthase (Pedersen, 1996; Weber and Senior, 1997). During ATP synthesis, the F_1 sector undergoes concerted conformational changes that couple modifications in nucleotide binding sites with the rotation of the γ-subunit. This concept of binding change mechanism (Boyer, 1993) has been strengthened by the crystal structure of the major part of the mitochondrial F_1 (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998). The affinity change of substrates and products at catalytic sites is coupled to proton transport by the rotation of the γ-subunit inside the α3β3 oligomer (Sabbert *et al.*, 1997; Zhou *et al.*, 1997; Noji *et al.*, 1997; Boyer, 1997). The ATP synthase thus operates as a rotary motor with a rotor consisting in subunits γ and ε of E. coli F_1 (Aggeler *et al.*, 1997). It has been proposed that the rotational catalysis also involves the rotation of the c-subunits relative to the single a-subunit in F_0 for completion of the proton pathway (Zhou *et al.*, 1997; Watts and Capaldi, 1997). As a consequence, subunits α, β, δ, a and b are probably all components of the stator in E. coli.

Whereas the E. coli ATP synthase contains eight different subunits, the bovine enzyme is composed of

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[2] Abbreviations: AMDA, 4-acetamido-4′maleimidylstilbene-2-2′disulfonic acid; ASIB, 1-(p-azidosalicylamido-4-(iodoacetamido) butane; F_0 andF_1, integral membrane and peripheral portions of ATP synthase; MPB, 3-(N-maleimidylpropionyl) biocytin; SDS, sodium dodecyl sulfate; su, subunit.

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sixteen (Collinson *et al.*, 1994). The Saccharomyces cerevisiae ATP synthase is composed of at least thirteen different subunits that are involved in the structure of the enzyme. The yeast F_0 part is composed of at least eight different subunits (Velours *et al.*, 1998).

Subunit f is one of the additional components (Collinson *et al.*, 1994; Walker and Collinson, 1994; Belogrudov *et al.*, 1996) whose gene inactivation leads to a defect in the assembly of the membrane sector in S. cerevisiae (Spannagel *et al.*, 1997). Data on the location of this subunit in yeast F_0 was provided by cross-linking experiments which revealed the proximity of the N-terminal portions of subunit 4 and subunit f (Spannagel *et al.*, 1998b). On the basis of the hydrophobic profile and prediction methods (Hofmann and Stoffel, 1993), the yeast subunit should display a unique potential transmembranous domain composed of amino acids 67 to 85. A similarity of only 21% was calculated between the bovine and the yeast subunits, but a similar potential transmembranous domain was found at the same locations. As a consequence the two proteins are likely the same. The data presented in this paper reinforce this hypothesis.

MATERIALS AND METHODS

Chemicals

ASIB[2] was from Pierce. AMDA and MPB were obtained from Molecular Probes. All other chemicals were of reagent grade quality. Oligonucleotides were purchased from Genset corporation.

Strains

The Saccharomyces cerevisiae strain D273-10B/A/H/U (MATα, met6, ura3, his3) was the wild type strain (Paul *et al.*, 1992). The mutants in subunit f 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 f were obtained by transformation of the SVY14 (MATα, met6, ura3, his3, ATP17::URA3) strain (Spannagel *et al.*, 1997) by the low copy shuttle vector pDR1 (Razaka *et al.*, 1994) bearing a 1980-bp BamH1-Sal1 DNA fragment containing the altered ATP17 gene. Single-stranded DNA 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 (Razaka *et al.*, 1994) and T7 DNA polymerase. Mutations were confirmed by DNA sequencing (Sanger *et al.*, 1977). The LiCl method (Ito *et al.*, 1983) was used to transform the null mutant SVY14 strain with the resulting plasmids. Transformants were selected and subcloned on minimal medium containing methionine and glucose as carbon source. Cells were grown aerobically at 28°C in a complete liquid medium containing 2% lactate as carbon source (Arselin de Chateaubodeau *et al.*, 1976) and harvested in logarithmic growth phase.

Biochemical Techniques

Mitochondria were prepared according to Guerin *et al.*, (1979). Protein amounts were determined according to Lowry *et al.*, (1951) in the presence of 5% SDS using bovine serum albumin as standard. The specific ATPase activity was measured at pH 8.4 according to Somlo (1968). Oxygen consumption rates were measured in the respiration medium (0.65 M mannitol, 0.3 mM EGTA, 3 mM Tris-phosphate, 10 mM Tris-maleate, pH 6.75) as described in (Rigoulet and Guerin, 1979). Variations in transmembrane potential ($\Delta\Psi$) were evaluated in the same medium by measurement of fluorescence quenching of rhodamine 123 with an SFM Kontron fluorescence spectrophotometer (Emaus *et al.*, 1986). Immunoprecipitated ATP synthases were prepared from 2 mg of mitochondrial proteins as in (Todd *et al.*, 1980). Polyclonal antibodies raised against the α-subunit were added to the 100,000 g supernatant of the 0.375% Triton X-100 mitochondrial extract. The immunoprecipitated proteins were washed with a buffer containing 1% Triton X-100 (w/v), 150 mM NaCl, 10 mM sodium phosphate pH 7.0. The final pellet was dried under vacuum and dissolved in 20 μl of dissociation buffer devoid of reducing agent. SDS-PAGE was according to Laemmli (1970) and Schagger and Von Jagow (1987). The slab gel was silver-stained according to Ansorge (1983). Western blot analyses were performed as described previously (Arselin *et al.*, 1996). Polyclonal antibodies raised against subunits f and 4 were used at a 1:10,000 dilution. ProBlott membranes were incubated with peroxidase-labeled antibodies at a dilution of 1:10,000 and revealed with the ECL reagent of Amersham International. MPB-labeling, AMDA-labelling and cross-linking experiments with heterobifunctional reagents were performed as described previously (Spannagel *et al.*, 1998b).

RESULTS

Tricine-SDS-PAGE of the Yeast ATP Synthase

The separation of the different components is shown in Figure 1. Our preparations are usually analysed with long slab gels that are made according to Laemmli (1970) or according to Schagger and Von Jagow (1987). The latter gives an efficient separation of proteins with molecular masses that are in the range of 3-10 kDa, but by contrast with what was observed with the Laemmli method (Arselin *et al.*, 1996), subunits 4 and 6 are not resolved, and the subunit h migrates faster than the δ-subunit, but at its expected molecular mass. The presence of loosely associated proteins is dependent on the concentration of Triton X-100 used during the extration and the purification steps. When all the purification steps are performed in the presence of 0.1% (w/v) Triton X-100, it is possible to keep the small subunits e and g associated to the complex (Vaillier *et al.*, 1999).

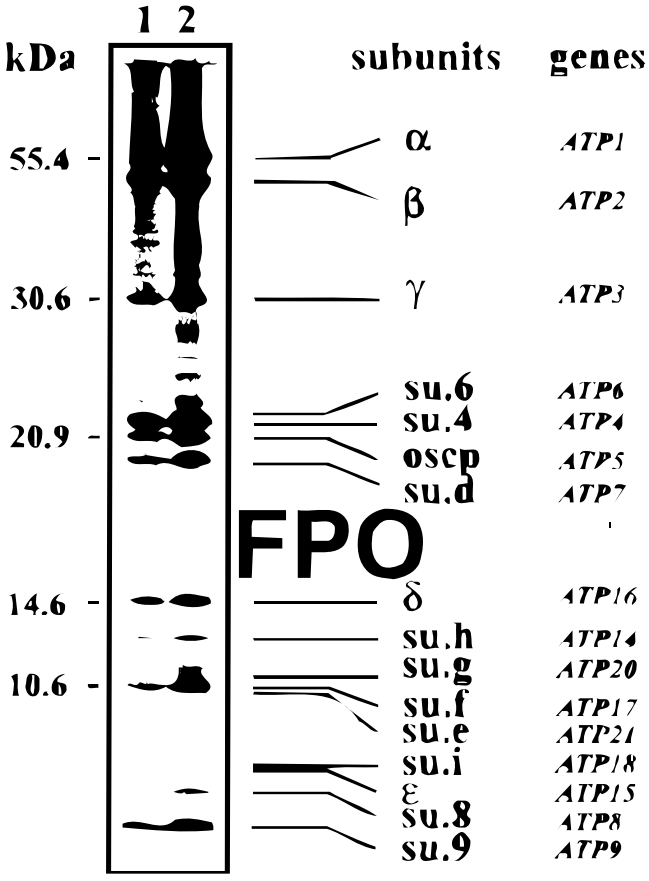

Fig. 1. SDS-polyacrylamide gel electrophoresis of yeast ATP synthase. Tricine-SDS-PAGE according to Schagger and Von Jagow (1987). Lanes 1 and 2: 16 and 32 μg protein, respectively. The slab gel was silver-stained.

Short Versions of Subunit f Complement the Null Mutant in ATP17 Gene

From prediction methods, the yeast subunit f appears as an integral inner membrane protein that crosses the membrane once from amino acid residues 67 to 85 (Fig. 2). A short hydrophilic C-terminus of 11 amino acid residues, which contains 4 histidines, should protrude from the membrane. From the 4 histidine residues, His88 and His95 are conserved in the bovine subunit. Western blot analysis of yeast mitochondrial membranes and purified ATP synthase showed that subunit f reacted highly with the Ni-nitrilotriacetic acid-peroxidase-conjugated reagent (not shown). Although the sequence of the last 11 amino acids was different from a histidine tag it appears that the 4 histidine residues of the C-terminus trap Ni^{++} ions. This could have meant that a potential cation binding site was involved in a regulation process. Three different strains having short versions of subunit f were constructed to clarify this (Fig. 2). The R87stop strain lacked H88, H89 and H95. The Y83stop strain also lacked H85. The W67stop strain had a version of subunit f that was devoid of the histidine region and of the predicted transmembranous domain. Phenotypic analysis of the 3 mutants revealed that they grew with lactate or glycerol as substrate (Table 1) whereas the null mutant did not (Spannagel *et al.*, 1997). However, an increasing doubling time was measured with respect to the increase in the C-terminal deletion of the subunit. The ATPase activities of R87stop and Y83stop mitochondria were not significantly modified, nor were their oligomycin sensitivities (Table 1). On the contrary, W67stop mitochondria had a two-fold decrease in specific ATPase activity that was in addition poorly oligomycin-sensitive. However, the ADP/O ratio for W67stop and Y83stop mitochondria was similar to that of the wild type with NADH as substrate, thus showing the same efficiency of both ATP synthases under physiological conditions (pH 6.75). The main difference between W67stop and the wild type mitochondria was a decrease in the respiration rates of the former. Y83stop mitochondria gave intermediary results between those of W67stop and wild type mitochondria. With NADH as respiratory substrate, the uncoupled respiration rate of W67stop mitochondria

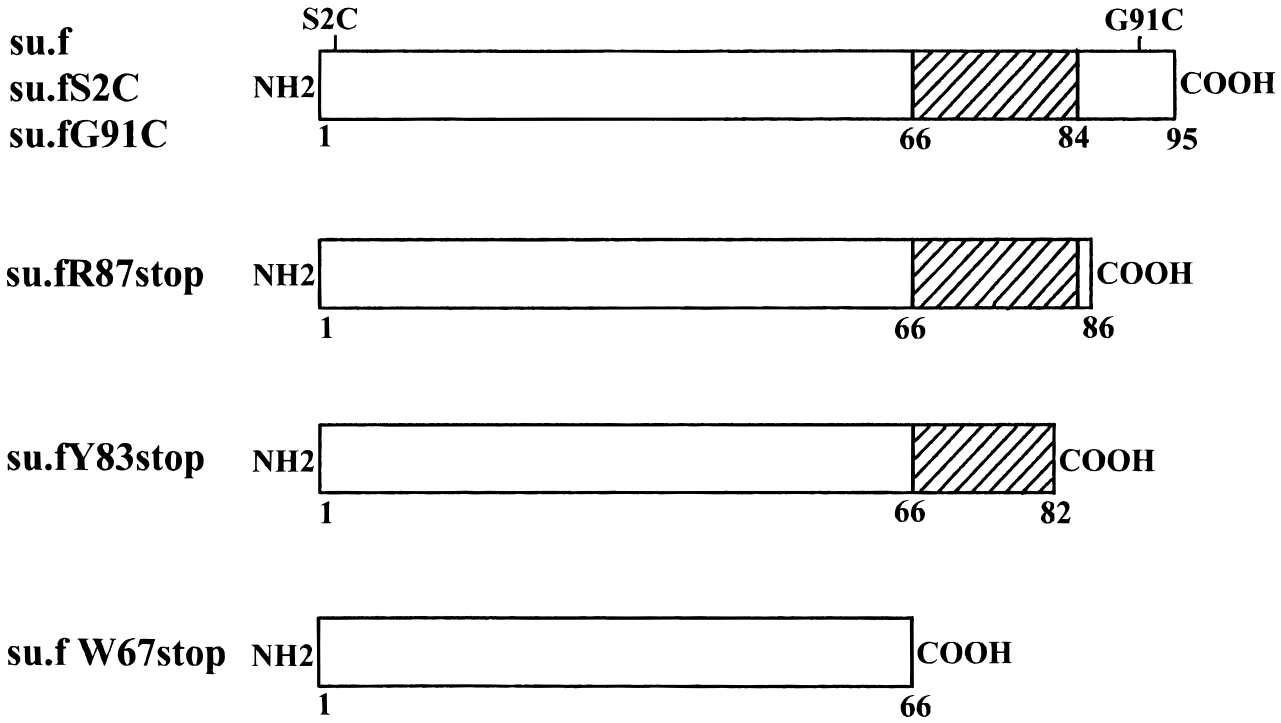

Fig. 2. Schema showing modified and truncated versions of subunit f. The wild type subunit f is composed of 95 amino acid residues. Su.fS2C, su.fG91C, su.fR87stop, su.fY83stop and su.fW67stop were constructed by using the oligonucleotide mutagenesis procedure as described in the Materials and Method section. The hatched area corresponds to the predicted transmembranous domain of subunit f. The Tmpred program of the ISREC (Swiss Institute for Experimental Cancer Research) was used (Hofmann and Stoffel, 1993).

was 75 % that of the wild type, thus revealing a decrease in the amount of respiratory chain component as is frequently observed in yeast mutants modified in ATP synthase (Ebner and Schatz, 1973; Paul *et al.*, 1989; Uh *et al.*, 1990; Tzagoloff and Dieckmann, 1990; Vaillier *et al.*, 1999).

The proton pumping activities of the mitochondrial preparations were measured in the same conditions as those used for the respiration rate measurements (Fig. 3). In the presence of ethanol, wild type mitochondria displayed a fluorescent quenching of rhodamine 123 which was transiently decreased

Table I. Phenotypic analyses of mutants in the ATP synthase subunit f

		ATPase activity			Respiration rates			
Strain	Generation Time	−Oligomycin	+Oligomycin	remaining activity	State 4	State 3	+CCCP	ADP/O
	min	nmol Pi/min/mg protein		%	nmol O/min/mg protein			
wild type	148	6075 ± 110	620 ± 55	10	395 ± 110	1020 ± 120	1615 ± 200	1.6 ± 0.1
su.fR87stop	161	6980 ± 85	830 ± 55	12	n.d.	n.d.	n.d.	n.d.
su.fY83stop	185	5965 ± 150	1055 ± 35	18	280 ± 40	900 ± 140	1470 ± 120	1.6 ± 0.1
su.fW67stop	204	2770 ± 150	1600 ± 220	58	210 ± 20	680 ± 20	1230 ± 140	1.6 ± 0.2

The doubling time was measured with cells grown on a complete medium containing 2% lactate. Turbidimetry was measured at 550 nm for the estimation of cell concentration after appropriate dilution. Mitochondria were prepared from yeast cells grown with 2% lactate as carbon source. Oxidative phosphorylation measurements were made with freshly prepared wild type and mutant mitochondria. Respiration rates were obtained with NADH as substrate. ATPase activities were measured at pH 8.4 with frozen and thawed mitochondria (Vaillier *et al.*, 1999). Results are the average of 4 different experiments and are presented with the standard deviation. Oligomycin (6 μg/mL) and CCCP (3 μM) were added where indicated.

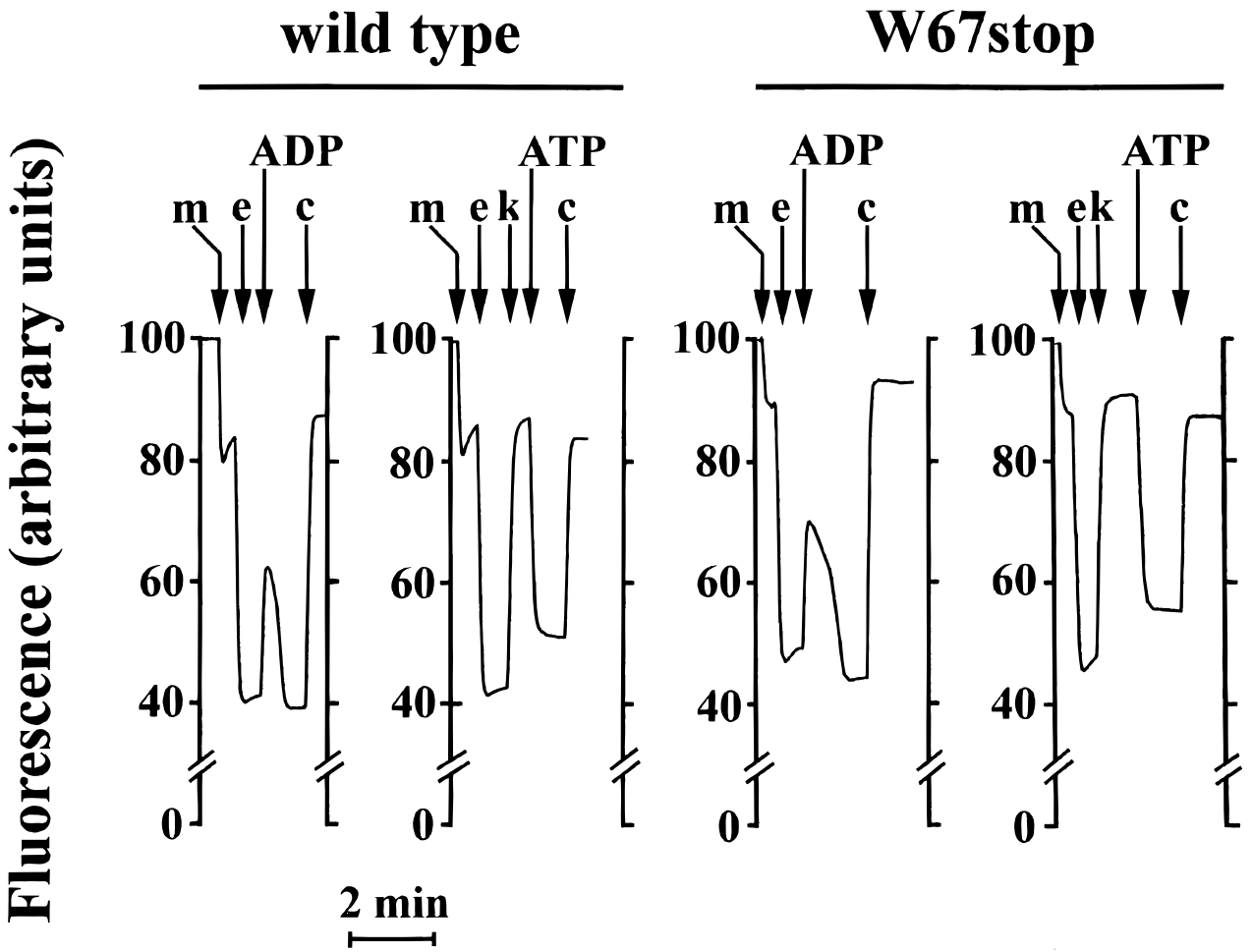

Fig. 3. Mitochondrial energization monitored by fluorescent quenching of rhodamine 123. D273-10B/A/H/U mitochondria (wild type) and W67stop mitochondria were incubated in 2 mL of respiration medium. Additions were 0.3 mg mitochondrial proteins (m), 10 μl of ethanol (e), 50 μM ADP, 3 μM CCCP (c), 200 μM KCN (k). One mM ATP was added 30 s after KCN addition.

with the addition of 50 μM ADP, thus reflecting a decrease in the transmembranous $\Delta\Psi$ due to a proton influx through F_0 during ADP phosphorylation. After ADP consumption, the $\Delta\Psi$ increased. Finally CCCP addition produced a reversal of the fluorescent quenching corresponding to the collapse of the $\Delta\mu H^+$. In the case of W67stop mitochondria, ADP addition induced the same decrease in fluorescent quenching of the dye but the time during which ADP was consumed was significantly increased, i.e., in agreement with a low respiration rate of W67stop mitochondria during the ADP phosphorylation state (State 3). Modifications of the transmembrane $\Delta\Psi$ mediated by the ATPase proton pumping activity were analyzed after energization of mitochondria by ethanol, an activation step which is necessary to remove the natural inhibitor IF1, which otherwise would inhibit the ATPase activity (Schwerzmann and Pedersen, 1986, Shouppe *et al.*, 1999). KCN addition collapsed the $\Delta\Psi$ by inhibiting proton pumping mediated by the respiratory chain. Subsequent ATP addition promoted a fluorescent quenching of the dye which was DCCD- and oligomycin-sensitive (Vaillier *et al.*, 1999), thus reflecting the proton pumping mediated by the ATPase. ATP-dependent proton pumping experiments showed a nearly similar fluorescent quenching of the dye for both types of mitochondria. Thus, the uncoupling observed at alkaline pH (8. 4) and indicated by a loss of oligomycin-sensitive ATPase activity was not observed in ATP-dependent proton pumping experiments.

It was important to investigate the presence or not of short versions of subunit f in the mutant strains.

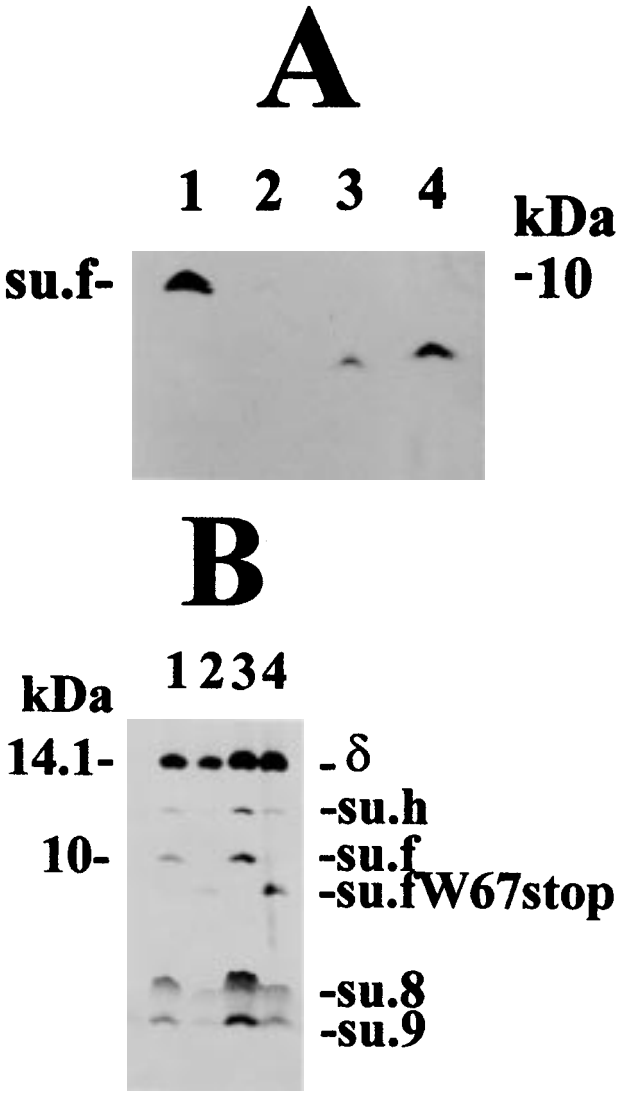

Fig. 4. Evidence of short versions of subunit f in the complemented yeast strains. A: Western blot analysis of total yeast cellular extracts. Cells were grown with 2% lactate as carbon source, harvested and cellular extracts were prepared according to Yaffe (1991). The SDS solubilized samples (70 μg protein) were submitted to a SDS-PAGE according to Schagger and Von Jagow (1987). The slab gel was transferred to a ProBlott membrane that was incubated with polyclonal antibodies raised against subunit f. Lane 1; wild type, lane 2; W67stop, lane 3; Y83stop, lane 4; R87stop. B: SDS-PAGE of immunoprecipitated ATP synthase. Two mg of mitochondrial proteins were dissolved in 0.375% Triton X-100. After centrifugation the ATP synthases were purified from the supernatant by immunoprecipitation with antibodies raised against the α-subunit. The washed pellets were dissolved in 20 μl of dissociation buffer. Lanes 1 and 3; wild type, 5 and 10 μl, respectively. Lanes 2 and 4; W67stop, 5 and 10 μl, respectively. The slab gel was silver-stained. su. = subunit.

Total yeast cell extracts were analyzed by Western blot. Su.fY83stop and su.fR87stop ran faster than the wild type su.f (Fig. 4) whereas su.fW67stop was not detected. If the C-terminus of subunit f were to contain the antigen site, it would explain the low intensity of su.fY83stop and su.fR87stop and the lack of detection of su. fW67stop. Finally, the presence of su.fW67stop in the mutant ATP synthase was shown by SDS-PAGE analysis of the immunoprecipitated complex. ATP synthases of W67stop mutant and wild type mitochondria were purified from a 0.375% Triton X-100 extract by addition of polyclonal antibodies raised against the α-subunit. The immunoprecipitation of the whole enzyme revealed a good assembly of F_1 and F_0 sectors which was not observed with the null mutant strain SVY14 (Spannagel *et al.*, 1997). In Figure 4B, only the lower part of the silver-stained slab gel corresponding to the migration range between the δ-subunit and subunit 9 is shown. The immunoprecipitates were washed with 1% Triton X-100 in order to remove subunit e which co-migrates with subunit f (Vaillier *et al.*, 1999). Su.fW67stop ran faster than the wild type subunit. From these results it appears that the first 67 residues of subunit f are sufficient to restore the function of the complex. Moreover, the last 28 amino acid residues including the postulated transmembranous domain are not essential for the binding of subunit f to other components of the complex. As a result, the N-terminal domain interacts tightly with other subunits of the enzyme because it remains bound to the complex under the washing conditions of the immunoprecipitate.

Labeling of the C-Terminus of Subunit f by Non-Permeant Maleimide Reagents

The orientation of the yeast ATP synthase subunit f was defined by using cysteine substitution mutants and non-permeant maleimide reagents. S2C and G91C mutants were constructed, thus giving unique targets to cysteine reagents as the wild subunit f is devoid of cysteinyl groups. The two mutants grew with glycerol as carbon source, thus showing that the oxidative phosphorylation was not altered (not shown). When the mitochondrial Triton X-100 extracts of both mutants were incubated with MPB or AMDA, the subunit f was modified as indicated by a decreased mobility on SDS-PAGE due to the binding of maleimide reagents (Fig. 5A). This points to the same accessibility of both cysteinyl groups in the absence of the membranous

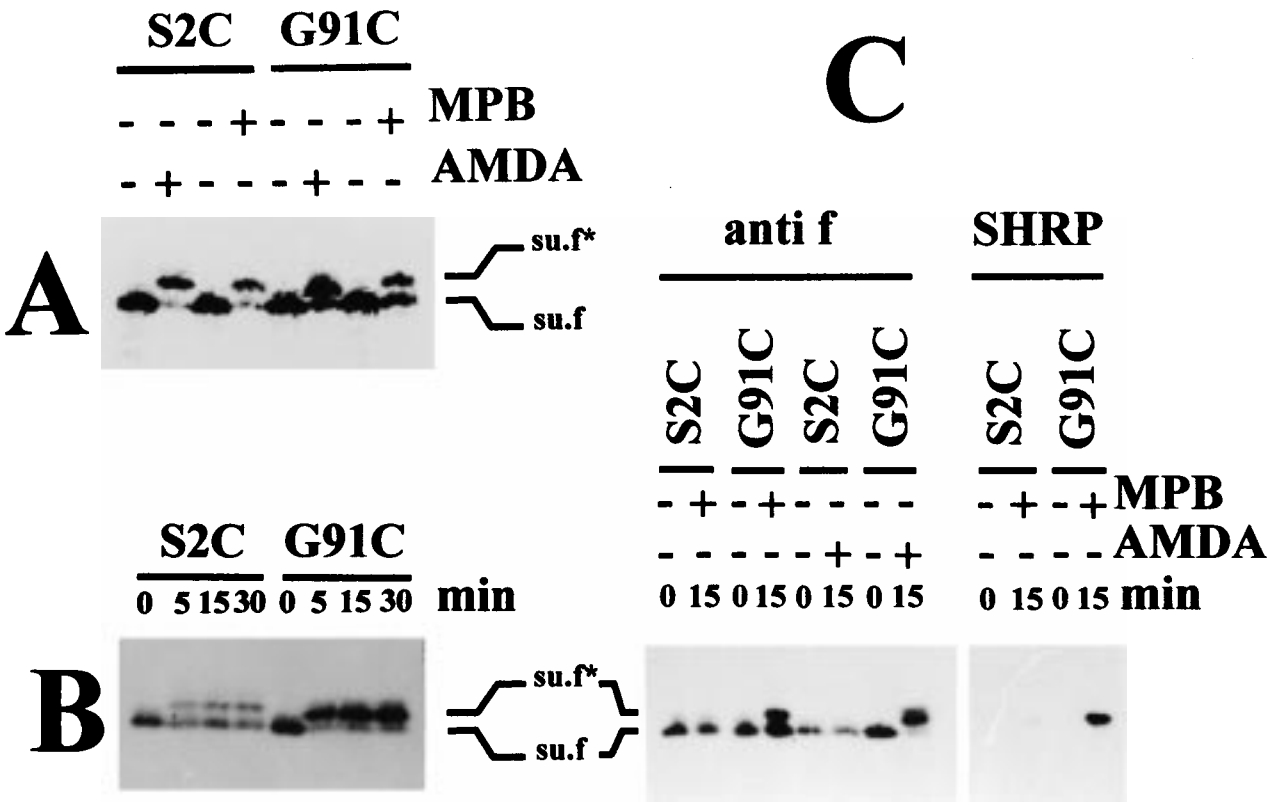

Fig. 5. Orientation of subunit f with respect to the yeast inner mitochondrial membrane. A: Western blot analysis of mitochondrial Triton X-100 extracts. Triton X-100 extracts (0.5 mg protein) were incubated or not for 15 min at 30°C with either 0.2 mM MPB or 3 mM AMDA. The reaction was stopped by addition of 60 mM of 2-mercaptoethanol. Proteins (30 μg) were separated by SDS-PAGE and transferred as in figure 4A. The blot was incubated with polyclonal antibodies raised against subunit f. The star (*) shows the modified subunit f. B: Western blot analysis of S2C and G91C mutant mitochondria labeled by AMDA. Thawed mitochondria (0.5 mg protein) were incubated for 5, 15 and 30 min with 3 mM AMDA at 30°C. The reaction was stopped by 2-mercaptoethanol addition and samples (30 μg protein) were analyzed as in figure 4A. C: Western blot analysis of intact S2C and G91C mutant mitochondria. Freshly prepared mitochondria (1 mg protein) were incubated for 15 min with either 3 mM AMDA or 0.2 mM MPB. The reaction was stopped and samples (20 μg protein) were analyzed in the left part of the figure as in figure 4A. In the right part of the figure, mutant ATP synthases were extracted by 0.375% Triton X-100 and purified by immunoprecipitation as in Fig. 4B. One tenth of solubilized immunoprecipitates was analyzed. The blot was incubated with streptavidin horse radish peroxidase (SHRP) at a dilution of 1:1,000.

barrier (the wild subunit f was not labeled by either maleimide reagent, not shown). A kinetic of the labeling was done with mitochondria prepared by the protoplast method and stored at −70°C after freezing in liquid nitrogen. The labeling was maximal for a 15 min incubation time at 30°C. Su.fG91C was highly modified by AMDA which was not the case for su.fS2C (Fig. 5B). An identical result was obtained with MPB (not shown). These data indicate a probable alteration of mitochondrial inner membrane integrity during freezing and thawing of mitochondria. To obtain clear results, fresh mitochondria were prepared and immediately incubated with the non-permeant maleimide reagents. In these conditions su.fS2C was not modified whereas su.fG91C showed a decrease in its electrophoretic mobility (Fig. 5C). The altered electrophoretic mobility of small proteins upon binding of the bulk maleimide reagents is an easy way to demonstrate their accessibility (Valiyaveetil and Fillingame, 1998; Long *et al.*, 1998; Spannagel *et al.*, 1998b). Another way is to identify the reagent itself. Streptavidin horseradish peroxidase reacted highly with the MPB-labeled su.fG91C (Fig. 5C), whereas no significant labeling was observed upon incubation of fresh S2C mitochondria with MPB. These results clearly indicate an intermembrane location of the C-terminus of subunit f and a matrix location of the N-terminus, which is in agreement with the data reported for the bovine subunit on the basis of proteolytic cleavages (Belogrudov *et al.*, 1996). In addition, these data reinforce the homology

of the two subunits and the existence of the unique transmembranous domain.

Cross-Linking of Subunits f and 4

Previous experiments have shown two cross-linked products that involve mutated subunits 4 at positions 54 or 55, and subunit f upon incubation of either mitochondria or Triton X-100 extracts with the heterobifunctional reagent ASIB (Spannagel *et al.*, 1998b). Experiments were done in order to confirm such a linkage from subunit f towards subunit 4 (Fig. 6). Western blot analyses of G91C Triton X-100 mitochondrial extract showed a 33 kDa band that reacted with polyclonal antibodies raised against subunits f and 4, whereas the S2C Triton X-100 extract displayed only a faint band at the same level (Fig. 6A). No other band was detected in the presence of ASIB. In the experiment with antibodies raised against subunit 4, the film was overexposed in order to detect the 4+f dimer, thus resulting in a darkness of the control sample (lane 1). The slight relative molecular mass difference observed between the su.4D54C+su.f dimer and the su.fS2C+su.4 dimer reflects a different mode of linking between the 2 subunits, which leads to different hydrodynamic volumes of the cross-linked products. When whole G91C mutant mitochondria were incubated with ASIB, the 33 kDa band was absent. This result indicates a poor accessibility of the azido group of ASIB to subunit 4 inserted in the membrane. Without incubation with ASIB, we noticed the presence of a 19 kDa band in the N-ethylmaleimide-treated G91C mutant mitochondria that reacted with anti-subunit f (Fig. 6B). This band was absent in the S2C mutant mitochondria (not shown). The 19 kDa band which is 2-mercaptoethanol-sensitive (not shown) could correspond to a dimerization of subunit f. Such a dimeriza-

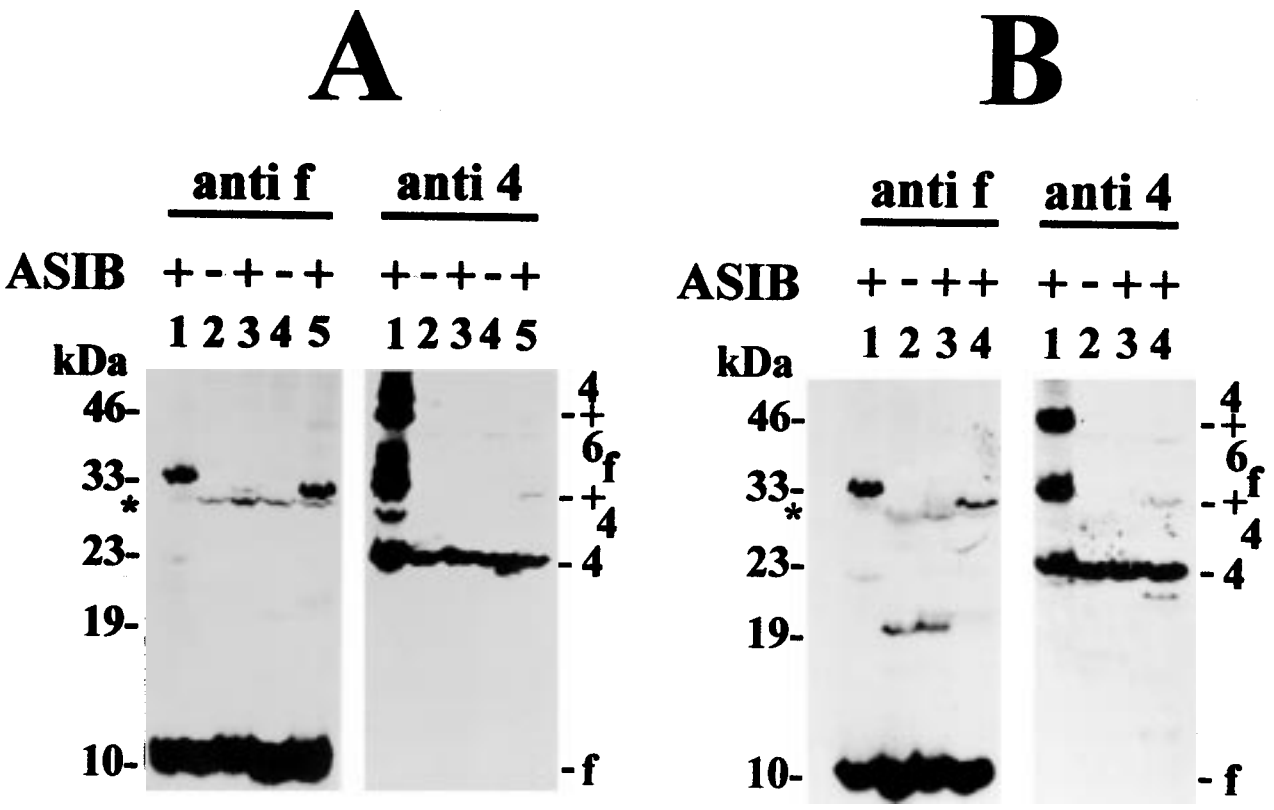

Fig. 6. Cross-linking from subunit f towards subunit 4. 0.375% Triton X-100 extracts were prepared from 1 mg of G91C mitochondrial protein. The strain having the D54C mutation in subunit 4 (Spannagel et al., 1998b) was used for comparison. 0.2 mL of extracts were incubated or not with 0.4 mM ASIB for 2 h in the dark at room temperature. After addition of 10 mM N-ethylmaleimide the samples were illuminated at 365 nm for 10 min. Proteins (30 μg) were submitted to a 15 % SDS-PAGE and transferred to a ProBlott membrane. The blots were incubated with polyclonal antibodies raised against subunits f and 4. An unknown protein that cross-reacts with the antiserum raised against subunit f is marked as *. A: Lane 1; (su.4)D54C mitochondria, lanes 2 and 3; S2C extracts, lanes 4 and 5; G91C extracts. B: Lane 1; (su.4)D54C extract, lanes 2 and 3; G91C mitochondria, lane 4; G91C extract.

tion of F_0 components has recently been reported for subunit 4 (Spannagel *et al.*, 1998a). In addition, a dimerization of the yeast enzyme has also been demonstrated by Arnold *et al.*, (1998) involving supernumerary subunits g and e. An heterodimer of su.fG91C and another component of 9–10 kDa having a cysteinyl group close enough to make a disulfide bond when the enzyme is inserted in the membrane is also an alternative hypothesis. This point is under investigation.

DISCUSSION

Subunit f is an essential component of the mitochondrial ATPase that has been found in the mitochondrial ATPases of bovine and S. cerevisiae. An open reading frame encoding a putative subunit f has been found in Schizosaccharomyces pombe chromosome II (EMBO accession number AL034433). The two yeast proteins display an identity of 41.1% in 95 amino acids. In S. cerevisiae, inactivation of the structural gene ATP17 leads to the loss of growth on non-fermentable substrates, but complementation of the null mutant by a low copy vector bearing the wild copy of subunit f restores the oxidative phosphorylations (Spannagel *et al.*, 1997). By using cysteine substitution mutants, we showed that the C terminal part of subunit f is in the intermembrane space on the basis of the accessibility of non-permeant maleimide reagents, whereas the N terminus of the protein is inaccessible in the presence of the membranous barrier. This result is in agreement with the data of Belogrudov *et al.*, (1996) who demonstrated the exposition of the carboxyl terminus of the bovine subunit f on the cytosolic side of the mitochondria by using trypsin and chymotrypsin action on the mitoplasts. This location is similar to that of the N terminus of subunit 6, and the hydrophilic loop of subunit 4 containing residues D54 and E55 (Spannagel *et al.*, 1998b). Such a location of the C terminus of subunit f rendered possible its cross-link with subunit 4, as shown by the 23 kDa cross-linked product obtained between su.4D54C and su.f upon incubation with the heterobifunctional reagent ASIB having an 18 A arm. A similar cross-linked product was obtained between su. fG91C and subunit 4 with ASIB but only when the complex was solubilized by Triton X-100. We have already reported that only a few amino acids (residues 54 and 55) of subunit 4 are accessible from the intermembrane space, the remainder of the molecule being directed towards the matrix space. As a consequence, it is likely that a steric hindrance occurs when the complex is integrated into the membrane. This is not in favour of a linking between the two subunits, while this is not the case with the Triton X-100 solubilized enzyme. Cross-linking of Su.fS2C Triton X-100 extract with ASIB resulted in only a faint band migrating at the same level as the su.fG91C + su.4 dimer. The low response with antibody raised against subunit 4 that is observed at the f+4 dimer level could explain the absence of the corresponding band in lane 3 of figure 6. Alternatively, the faint band could correspond to another component of the enzyme such as OSCP or subunit d which have close molecular masses to that of subunit 4. Anyway, such a low response indicates that although accessible to maleimide reagents in the absence of the membranous barrier, position 2 of subunit f is far from other components of the ATP synthase.

The protrusion of the C terminus of subunit f into the intermembrane space and the presence in this part of the subunit of 4 histidine residues of which 2 are conserved in the bovine subunit was initially thought to have a physiological role like a cation binding site. Mutant strains having a low copy vector bearing short versions of subunit f devoid of histidine residues grew with glycerol or lactate as substrate, thus indicating that these amino acids are not essential in our experimental conditions. Also, the removal of the postulated transmembranous domain of subunit f slightly affected growth with non-fermentable substrates. As the mitochondria of such a mutant displayed an oxidative phosphorylation efficiency similar to that of the wild type, we concluded that this short version of subunit f was able to complement the null mutant strain. However, extreme conditions like ATPase activity measured at pH 8.4 led to a decrease in oligomycin-sensitive ATPase activity, thus indicating a functional uncoupling between the catalytic and membranous sectors. As a result, the whole subunit f is involved in the stability of the complex. We hypothesize that the decrease in specific ATPase activity for W67stop mutant is most likely the result of a decrease in the stability of the complex which affects its biogenesis and which in turn affects the assembly of the other respiratory chain complexes.

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