Topography of the Yeast ATP Synthase F_0 Sector by Using Cysteine Substitution Mutants. Cross-Linkings between Subunits 4, 6, and f[†]

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Received June 23, 1997; Revised Manuscript Received September 24, 1997[⊗]

ABSTRACT: The arrangement of the N-terminal part of subunit 4 (subunit b) has been studied by the use of mutants containing cysteine residues in a loop connecting the two N-terminal postulated membrane-spanning segments. Labelling of the mutated subunit 4 by the fluorescent probe *N*-(7-(dimethylamino)-4-methyl-3-coumarinyl)maleimide revealed that the sulfhydryl groups were modified upon incubation of intact mitochondria. In addition, the nonpermeant sulfhydryl reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid prevented the 3-(*N*-maleimidylpropionyl)biocytin labeling of subunit 4D54C, thus showing a location of this residue in the intermembrane space. Cross-linking experiments revealed the proximity of subunits 4 and f. In addition a disulfide bridge between subunit 4D54C and subunit 6 was evidenced, thus demonstrating near-neighbor relationships of the two subunits and a location of the N-terminal part of the mitochondrially-encoded subunit 6 in the intermembrane space.

The mitochondrial ATP synthase is the major enzyme responsible for aerobic synthesis of ATP. ATP synthase exhibits a tripartite structure consisting of a headpiece (catalytic sector), a basepiece (membrane sector), and a connecting stalk. However, the enzyme resolves into only two parts. (i) The catalytic sector F_1, with subunits α, β, γ, δ, and ϵ, is a water-soluble unit retaining the ability to hydrolyze ATP when in a soluble form. (ii) The F_0 sector which is a detergent-soluble unit that is embedded in the membrane is composed of hydrophobic subunits forming a specific proton pathway. The 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 (*1*, *2*). The bovine enzyme is composed of sixteen different subunits (*3*) (IF1 included), whereas the *Escherichia coli* enzyme contains eight different subunits. The main difference between eukaryotic and prokaryotic enzymes resides in the presence of numerous additional subunits in the F_0 complex. The eukaryotic F_0 sector contains not only the three subunits a, b,and c like the *E. coli* enzyme but also subunits d, OSCP, A6L, and F6 (*4*, *5*). In addition, small polypeptides: subunits e, f, and g have recently been identified in the bovine enzyme (*3*, *6*).

We are at present studying the structure of the *Saccharomyces cerevisiae* ATP synthase which is composed of at least thirteen different subunits. The F_0 part contains at least eight different subunits. Three of them are mitochondrially encoded (subunits 6, 8, and 9) (*7*). The other five are subunit 4 (*8*), OSCP (*9*) subunit d (*10*), subunit h (*11*), and subunit f (*12*). Subunit 4 is homologous to the b-subunit of beef heart mitochondria (*13*). The structure of the eukaryotic b-subunit is composed of two domains: the N-terminal part is predominantly hydrophobic, and the C-terminal part is charged and hydrophilic. This subunit is considered to traverse the membrane twice via two hydrophobic stretches of amino acids, with N- and C-terminal parts emerging from the membrane on the F_1 side (*3*). From the analysis of yeast mutants, Paul et al. (*14*) have shown that the C-terminal part of subunit 4 is involved in the assembly of F_1 to F_0. On one hand, Houstek et al. (*15*) and Zanotti et al. (*16*) have shown that the bovine subunit is involved in proton conduction through F_0. On the other hand, site-directed mutagenesis of the N-terminal part (L68R,V69E) modified the hydrophobic character of the transmembrane domain of the yeast subunit. This modification destabilized the structure of the proton channel as shown by an energy coupling impairment due to proton-dissipating pathways through F_0 (*17*). As a result, like its counterpart subunit b in *E. coli* (*18*, *19*), subunit 4 appears necessary for a tight coupling between proton flux and ATP synthesis.

This study was designed to examine the structure of the N-terminal part of the eukaryotic subunit 4 (subunit b), and particularly to localize a short hydrophilic loop containing the amino acid residues 46−56 (*20*) and connecting the two postulated hydrophobic stretches of amino acids. Advantage was taken from yeast mutants in which cysteines were introduced into subunit 4 by site-directed mutagenesis. Labeling of cysteine residues by hydrophobic and hydrophilic maleimides provided a way to determine their location. In addition, the cysteine residues were used as targets to identify neighboring subunits to the loop.

[†] This research was supported by the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de l'Enseignement supérieur, the Université de Bordeaux II, and the Etablissement Public Régional d'Aquitaine. C.S. holds a research grant from the Ministère de la Recherche et de la Technologie.

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[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1997.

S0006-2960(97)01497-9 CCC: $15.00
Published on Web 01/13/1998

EXPERIMENTAL PROCEDURES

Materials. APA-Br,[1] DACM, and oligomycin were purchased from Sigma. APDP and ASIB were from Pierce. AMDA and MPB were obtained from Molecular Probes. All other chemicals were of reagent grade quality.

Yeast Strains and Nucleic Acid Techniques. The *S. cerevisiae* strain D273-10B/A/H/U (MATα, *met6, ura3, his3*) (*14*) was the wild type strain. Wild type control and mutants strains were obtained after complementation of the deleted−disrupted yeast strain PVY10 (MATα, *met6, ura3, his3*, ATP4::URA3) (*14*) by the low-copy shuttle vector pDR1 (*21*) containing the wild type ATP4 gene and the mutated versions of ATP4 gene, respectively. Mutants are named as (wild type residue)(residue number)(mutant residue) where the residues are given in the single-letter code.

The 1580-bp *Eco*RI−*Sal*I DNA fragment containing the wild-type ATP4 gene (*14*) was inserted into the polylinker of the shuttle vector pDR1. 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 AmpR-oligonucleotide (*21*) and T7 DNA polymerase. Mutations were confirmed by DNA sequencing (*22*). The LiCl method (*23*) was used to transform the deleted-disrupted PVY10 strain with the resulting plasmids. Transformants were selected and subcloned on minimal medium containing methionine and glucose as carbon source. The mutant PVY219 strain containing the chromosomal copy of the gene encoding for the subunit 4D54C was obtained by homologous recombination of the PVY10 strain with the 1580-bp *Eco*RI−*Sal*I DNA fragment containing the altered ATP4 gene. The transformants were selected by their ability to grow with lactate as carbon source. Phenotypic analysis of PVY219 clones showed that they recovered the uracil auxotrophy of the wild type strain.

Biochemical Procedures. Cells were grown aerobically at 28 °C in a complete liquid medium containing 2% lactate as carbon source (*24*) and harvested in logarithmic growth phase. Mitochondria were prepared according to Lang et al. (*25*) and Guérin et al. (*26*). Protein amounts were determined according to Lowry et al. (*27*) in the presence of 5% SDS. Bovine serum albumin was used as standard protein. The mitochondrial ATPase activity was performed at pH 8.4 (*28*). The specific ATPase activity of the purified F_1F_0 ATP synthase was measured in the presence of exogenous phospholipids (*11*). The crude mitochondrial Triton X-100 extracts were prepared from 1 mg of mitochondrial protein: the mitochondrial pellet was suspended in 0.1 mL of 4 mM Tris/acetate, pH 7.4, and incubated with 0.1 mL of 0.75% Triton X-100 (w/v) for 20 min at 4 °C. The mixture was then centrifuged at 109000*g* for 15 min at 4 °C. The ATP synthase was purified from the supernatant by adding 200 μL of serum containing rabbit polyclonal antibodies raised against the α-subunit. Immunoprecipitation was performed overnight at 4 °C, and the pellet was washed (*29*).

DACM-Labeling Experiments. Intact mitochondria (1 mg of protein) were centrifuged for 5 min at 15000*g*. The pellet was gently suspended in 0.1 mL of the isolation buffer consisting of 0.6 M mannitol, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM Tris/maleate, and the pH was adjusted to 7.4. The suspension was incubated for 30 min at 4 °C with 0.7 mM DACM. The reaction was stopped by adding 0.14 M β-mercaptoethanol. Mitochondria were washed three times by centrifugation (5 min, 15000*g*) with 0.6 M mannitol, 0.5 mM EGTA, 10 mM Tris/maleate, pH 6.8, and the ATP synthases were immunoprecipitated. The precipitate was dried, solubilized with 5% SDS and submitted to SDS−PAGE. Half of the sample was loaded per well.

MPB-Labeling and AMDA Protection. Freshly prepared mitochondria (1 mg of protein) were suspended in 0.1 mL of isolation buffer (pH 7.4) and incubated at 30 °C with either 200 μM MPB prepared as a 1 mM stock solution in the previous buffer or 3 mM AMDA. Protection of MPB binding by AMDA was done by preincubation with 3 mM AMDA followed by washing and incubation in the presence of 200 μM MPB. The reactions were stopped upon addition of 45 mM β-mercaptoethanol. Mitochondria were washed twice with the isolation buffer and centrifuged (10 min, 12000*g*). Finally, ATP synthase immunoprecipitates were prepared and analyzed by Western blotting. Blots were reacted either with streptavidin horse radish peroxidase with a 1:1000 dilution or antiserum raised against subunit 4 with a 1:10000 dilution.

Cross-Linking Experiments. Intact mitochondria (1 mg of protein in 0.1 mL of 0.6 M mannitol, 0.5 mM EGTA, 10 mM Tris/maleate, pH 7.4) and crude mitochondrial Triton X-100 extracts were incubated with ASIB, APDP, or APA-Br in the dark for 2 h at room temperature. Reactions with ASIB and APA-Br were stopped upon addition of 45 mM β-mercaptoethanol. The samples were then illuminated at 365 nm for 10 min at room temperature, dissociated with SDS, and analyzed by SDS−PAGE and Western blotting. F_1F_0 ATP synthase (50 μg of protein in 0.1 mL of 0.1% Triton X-100, 62.5 mM sucrose, 1 mM EDTA, 20 mM Tris/acetate, pH 7.5) was cross-linked with either APDP as above or 1.5 mM Cu(II)(1-10-phenanthroline)$_3$ for 1 h at 4 °C (*31*, *32*). The last reaction was stopped by addition of 10 mM EDTA.

SDS−PAGE and Western Blot Analyses. SDS−PAGE was performed as described previously (*8*). The slab gel was washed with methanol/water/acetic acid (5:5:1) for 15 min. Fluorescent bands were photographed under ultraviolet light, and the slab gel was silver-stained (*30*). Two-dimensional gel electrophoresis was as follows. The first

[1] Abbreviations: ϵ-ACA, ϵ-amino-*n*-caproic acid; AMDA, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; APA-Br, p-azidophenacyl bromide; APDP, *N*-[4-*p*-azidosalicylamido)butyl]-3′-(pyridyldithio)-propionamide; ASIB, 1-(*p*-azidosalicylamido)-4-(iodoacetamido)butane; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DACM, *N*-(7-(dimethylamino)-4-methyl-3-coumarinyl)maleimide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; pAB, *p*-aminobenzamidine dihydrochloride; F_0 and F_1, integral membrane and peripheral portions of ATP synthase; MPB, 3-(*N*-maleimidylpropionyl)biocytin. OSCP, oligomycin sensitivity conferring protein; SDS, sodium dodecyl sulfate; su, subunit.

[2] This experiment was performed with the yeast strain PVY219 whose D54C mutation was borne by the ATP4 gene at its chromosomal locus. This strain was constructed in order to obviate eventual artefacts caused by the low-copy vector bearing the modified version of the ATP4 gene. The specific ATPase activity of PVY219 mitochondria was similar to that of the wild type D273-10B/A/H/U as well as the oligomycin sensitivity. However, PVY219 cells displayed an increased doubling time with lactate as carbon source. This result has not been explained so far.

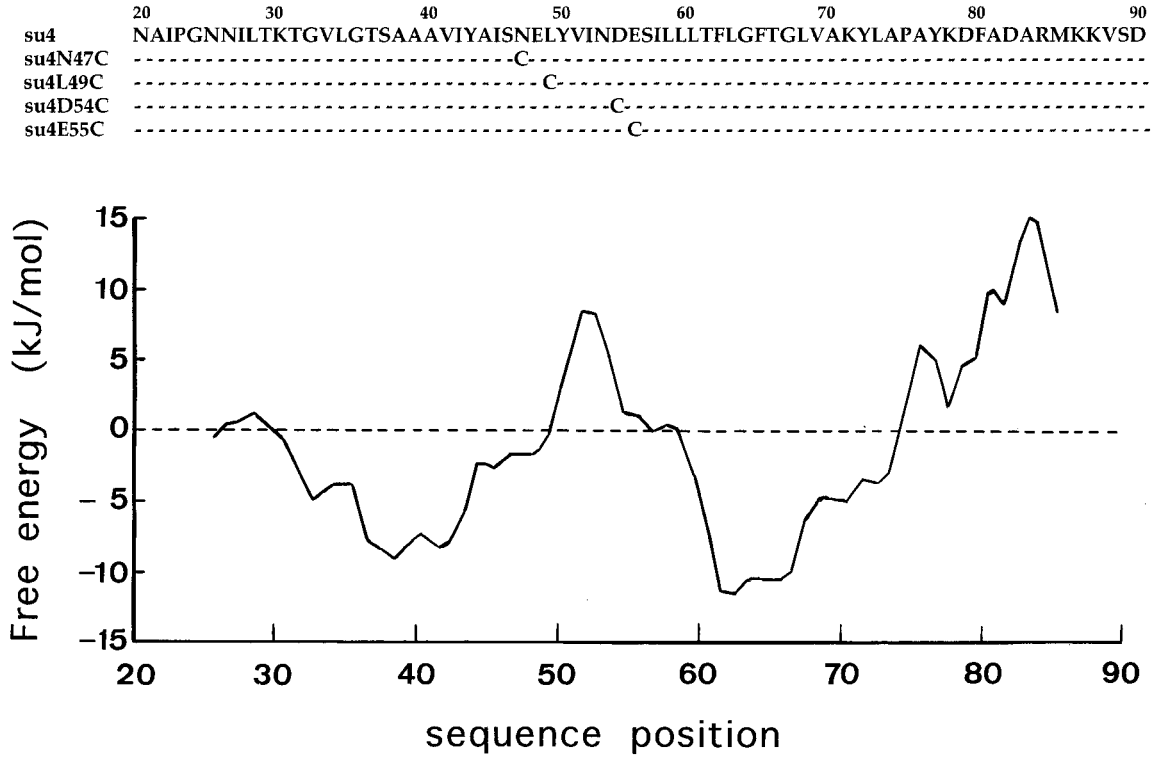

FIGURE 1: Hydrophobic profile of the N-terminal part of the wild type subunit 4. Hydrophobic profile was calculated according to Von Heijne (*47*). A window of 10 amino acids was used. The area below the dotted line indicates increased probability for a location in the hydrophobic lipid bilayer. A part of the amino acid sequence of subunit 4 and the mutations are shown at the top of the figure.

electrophoresis was performed on a rod gel (12 cm × 0.35 cm i.d.) containing a 15% running gel and a 7.5% stacking gel. After migration at 1 mA the rod gel was recovered and incubated for 20 min at 65 °C in the sample buffer containing 2% β-mercaptoethanol. The rod gel was then sealed with a 12% stacking gel on the top of a 13−18% acrylamide linear gradient. After migration the slab gel was silver-stained as above.

Rabbit antiserum to subunit f was obtained using an homogeneous preparation of yeast subunit f as antigen (*12*). Western blot analyses were described previously (*11*). Antibodies raised against subunits 4 and f were used with a 1:10000 dilution.

RESULTS

We generated yeast strains showing different versions of subunit 4 characterized by mutations in a loop region (residues 46−56) connecting the two N-terminal postulated membrane-spanning segments (*13, 20*) (Figure 1). The location of this loop was investigated by using four cysteine substitution mutants, each containing a sole cysteine residue in subunit 4 at locations 47, 49, 54, and 55. The mutant strains grew with lactate as carbon source, thus showing that the phosphorylation process was not altered. Furthermore, the mitochondrial oligomycin-sensitive ATPase activities were not significantly modified (Table 1).

Cysteine Labeling by Maleimides

The accessibility of cysteines was investigated by using the fluorescent hydrophobic reagent DACM, which forms a stable, strongly fluorescent adduct with thiol groups (*33*). In a preliminary experiment, DACM was reacted with crude mitochondrial Triton X-100 extracts. The ATP synthases were purified by using an immunoprecipitation procedure (*29*), and subunits were separated by SDS−PAGE. Subunits α, γ, and δ of the F_1 sector, OSCP, and subunits 6, 9, and the four mutated subunits 4 of the F_0 sector bound DACM, wheareas the wild type subunit 4 did not bind the maleimide (not shown). This result is in agreement with the fact that all these subunits (except the wild type subunit 4) display one endogenous cysteine residue (*9, 34−39*). Therefore, these cysteine residues are available for modification by the hydrophobic maleimide in the solubilized native ATP synthase, thus showing that these targets are not buried in the complex.

The accessibility of the cysteine residues of mutated subunits 4 was examined by incubation of intact mitochondria with DACM. ATP synthases were extracted, purified

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

strains	doubling time (min)	ATPase activity [(μmol of P_i min^{-1}) (mg of $protein^{-1}$)]	
		no addition	oligomycin
D273-10B/A/H/U	154	5.7 ± 0.2	0.5 ± 0.1
PVY219	248	5.2 ± 0.1	0.9 ± 0.1
wild type control	198	6.1 ± 0.1	0.6 ± 0.1
N47C	225	4.9 ± 0.3	0.6 ± 0.1
L49C	261	6.7 ± 0.1	1.5 ± 0.1
D54C	294	5.7 ± 0.3	1.3 ± 0.1
E55C	333	6.3 ± 0.1	1.1 ± 0.1

[a] Growth was monitored by turbidimetry at 600 nm. The growth rate was calculated in the exponential growth phase over a 10-h period. Yeast cells were grown at 28 °C with 2% lactate as carbon source. Mitochondria were isolated. ATPase assays were performed at 30 °C with addition of 6 μg of oligomycin per mL where indicated. The wild type control strain was the PVY10 strain complemented by the low-copy vector pDR1 bearing the wild type ATP4 gene.

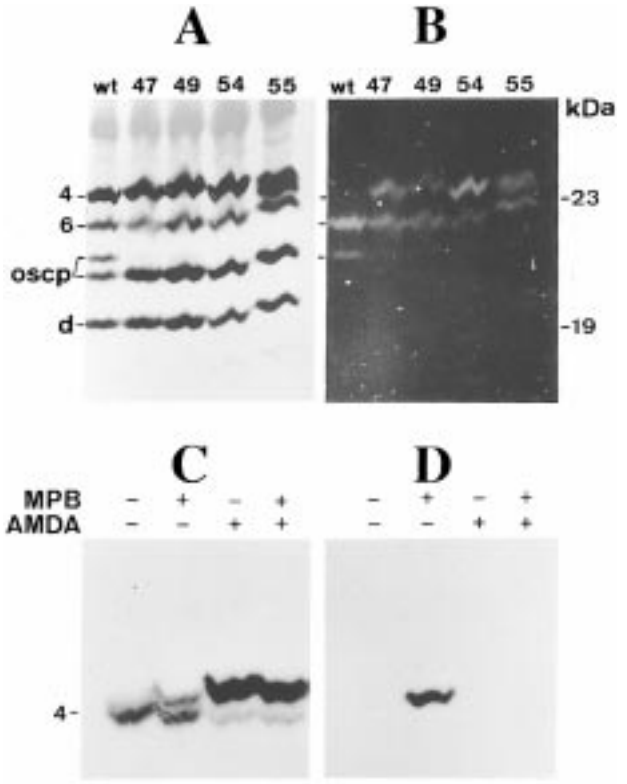

FIGURE 2: Labeling of subunit 4. (A, B) SDS−PAGE of wild type control and mutant immunoprecipitated ATP synthases isolated from intact mitochondria labeled by DACM. (A) Silver-staining. (B) Analysis for fluorescence under ultraviolet light. wt, wild type control (no mutation). The numbers at the top of the figure refer to the location of the mutations in subunit 4. (C, D) Western blot analyses of D54C-immunoprecipitated ATP synthases isolated from intact D54C mitochondria labeled by MPB and AMDA (see Experimental Procedures). Blots were revealed either with antibody raised against subunit 4 (C) or with streptavidin horseradish peroxidase (D).

as above and analyzed by SDS−PAGE. Of all the putative DACM-labeled subunits, only subunit 6, su4N47C, su4L49C, su4D54C, and su4E55C were fluorescent. Figure 2 shows the migration range of subunits 4 and 6, OSCP, and subunit d. In this experiment OSCP of control mitochondria was slightly labeled. As OSCP is a part of the stalk of the enzyme and is located in the matrix compartment, it appears that the wild type mitochondrial preparation was leaky. The DACM-bound OSCP migrated more slowly than the free subunit (Figure 2A). As a consequence, the absence of such an additional band in other samples was an indication of mitochondrial integrity. DACM-labeling of wild type mitochondria was repeated and confirmed the lack of accessibility of OSCP in freshly isolated and intact control mitochondria.

As DACM is a hydrophobic reagent, more definite evidence for the location of the hydrophilic loop was provided by blocking sulfhydryl groups with water-soluble maleimides. Freshly isolated and intact mitochondria were reacted with MBP and AMDA. The water-soluble maleimides MBP and AMDA labeled su4D54C and to a lesser extent su6C23 (not shown), whereas su4N47C and su4E55C were not accessible (L49C mutant was not tested). This was shown by an increase in the relative molecular mass of subunit 4 (Figure 2C). AMDA protected su4D54C from MPB-binding (Figure 2C-D), thus showing that the two maleimides were competitive toward the sulfhydryl group of Cys54. Subunit 6 was poorly labeled by 200 μM MPB, but higher concentrations of MPB were not assayed because of the low solubility of the reagent in aqueous solutions (not shown). From these data, we propose that the hydrophilic loop containing the amino acid residues 46−56 is directed toward the mitochondrial intermembrane space. Moreover, hydrophilic maleimides demonstrate more precisely that Cys54 of subunit 4 is exposed at the outer surface of the inner mitochondrial membrane. In addition, the fluorescent labeling of the sole cysteine (Cys23) of mature subunit 6 is also in favor of an intermembrane space location of the N-terminal part of subunit 6, as proposed previously (*40*).

Cross-Linking of Subunits 4 and f

The mutated subunits 4 were used for determining neighboring proteins in F_0. In a preliminary experiment, crude mitochondrial Triton X-100 extracts of the four mutants were reacted with ASIB and exposed to UV light. Two cross-link products migrating with relative molecular weight of 33 000 and 46 000 were found in D54C extract but not in N47C extract. L49C and E55C mitochondrial extracts contained only the 33 kDa product (not shown). The identification of the two neighboring proteins was undertaken from the D54C mitochondria. As subunit 4 has a molecular mass of 23,250 Da, the two neighboring proteins displayed molecular masses of 10 and 23 kDa, respectively.

Similar cross-links were obtained by incubation of intact mitochondria with ASIB. Wild type subunit 4 was not modified by the reagent, whereas three additional bands with relative molecular masses of 50 000, 46 000, and 33 000 were recognized by our anti-su4 antibodies in D54C mitochondria (Figure 3). The 50 kDa band was detected without incubation with the cross-linking reagent. This band was present only in D54C and E55C mitochondria. It disappeared upon reduction with 2% β-mercaptoethanol and was insensitive to a 10 mM NEM incubation prior to dissociation (not shown). In addition, the 50 kDa band was weakly extractible by Triton X-100 concentrations used to extract ATP synthase. Experiments are under way to identify this product.

Polyclonal antibodies raised against most yeast F_0 subunits were tested. We found that the 33 kDa product was a heterodimer of subunits 4 and f (Figure 3), which is in agreement with the sum of the respective molecular masses.

Cross-linking experiments of crude D54C mitochondrial Triton X-100 extracts with APA-Br, APDP and ASIB are presented in Figure 4. Increasing concentrations of reagents gave cross-link products from at least 20 μM. The small apparent mass increase of su4D54C should be the result of the binding of the reagent to the sulfhydryl group. The cleavable reagent APDP displayed similar cross-links to ASIB, and the linkages were fully reduced upon β-mercaptoethanol addition. The band of 46 kDa was obtained with the three reagents, but subunits 4 and f were not cross-linked by APA-Br which has the shorter arm (9 Å) (Figure 4A).

Cys54 of Subunit 4 Bound Subunit 6 by a Disulfide Bridge

Oxidation of crude D54C mitochondrial Triton X-100 extracts and intact D54C mitochondria by cupric 1−10 phenanthrolinate also gave the immunoreactive band of 46

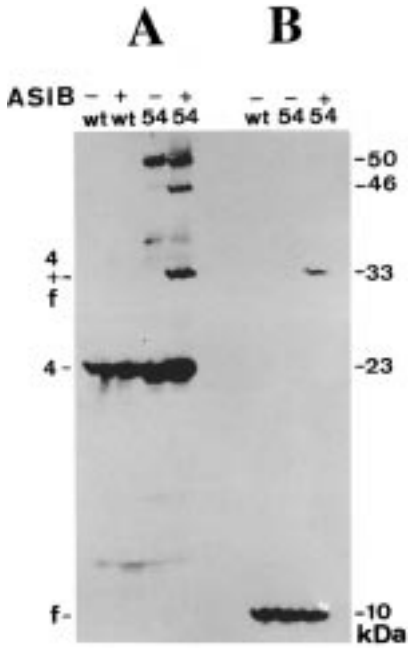

FIGURE 3: Cross-linking between subunits 4 and f. Western blot analysis of mitochondrial proteins (30 μg). Yeast mitochondria were incubated with ASIB in the dark for 2 h at room temperature. The reaction was blocked with β-mercaptoethanol, and samples were illuminated at 365 nm for 10 min and dissociated. Blots were revealed with antisera raised against subunit 4 (A) and subunit f (B). wt, wild type control (no mutation). The number 54 at the top of the figure refers to the location of the mutation in subunit 4.

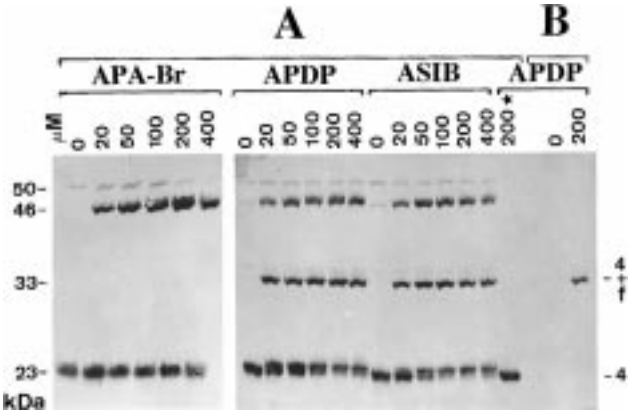

FIGURE 4: Cross-linkings with APA-Br, APDP, and ASIB. Western blot analysis of crude D54C mitochondrial Triton X-100 extract (30 μg of protein). The Triton X-100 extract was incubated with increasing concentrations of the three reagents as described in Figure 3. After dissociation, electrophoresis, and transfer, blots were reacted with antisera raised against subunit 4 (A) and against subunit f (B). *The sample was incubated with 200 μM APDP, submitted to irradiation and dissociated in the presence of 2% β-mercaptoethanol before electrophoresis.

kDa. This band was present in high amounts in the purified D54C F_1F_0 ATP synthase (Figure 5A, lane 1). Because anti-OSCP and anti-subunit d antibodies did not react with the 46 kDa cross-linked product, we took the disulfide bond formation between su4D54C and the cysteine residue of another yeast F_0 subunit to identify the neighboring subunit. Purified D54C ATP synthase was incubated under oxidative conditions. The intensity of the 46 kDa cross-linked product increased under oxidative conditions and disappeared under reducing conditions (Figure 5A). In Figure 5B the subunits of a $CuCl_2$-induced cross-linked D54C ATP synthase were

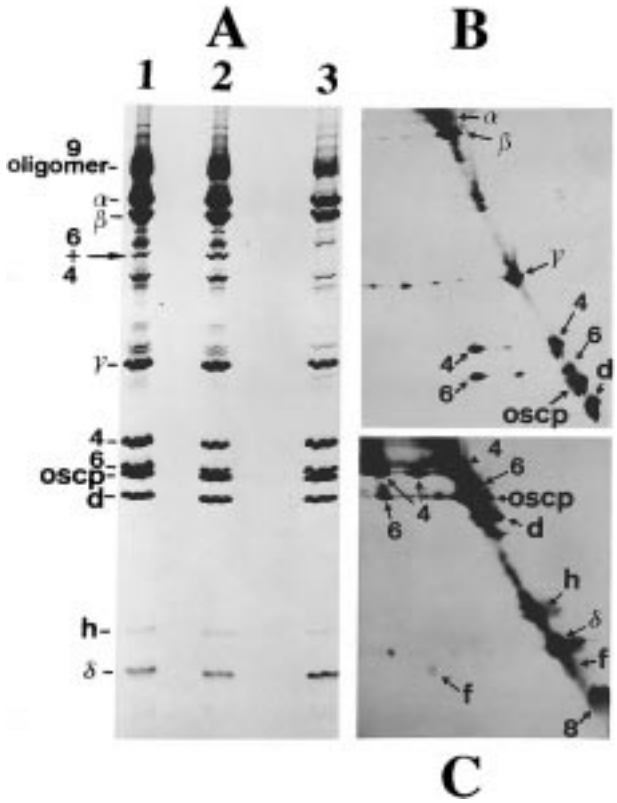

FIGURE 5: Disulfide bridge between subunit 6 and su4D54C. (A) SDS−PAGE of D54C ATP synthases (8 μg) incubated without (lane 1) or with 1.5 mM $CuCl_2$ (lanes 2 and 3). Before dissociation and electrophoresis, 2% β-mercaptoethanol was added to the sample in lane 3. The slab gel was silver-stained. The arrow indicates the 4−6 cross-link product. (B, C) Two-dimensional gel electrophoresis of D54C ATP synthase. (B) Purified enzyme (18 μg) was incubated with 1.5 mM $CuCl_2$ and submitted to 15% SDS−PAGE without β-mercaptoethanol treatment (first dimension from left to right); second dimension, 13−18% gradient of SDS−PAGE. The second dimension was done after incubation of the rod gel with 2% β-mercaptoethanol (see Experimental Procedures). (C) A crude D54C mitochondrial Triton X-100 extract was cross-linked with 200 μM APDP. The ATP synthase was immunoprecipitated and analyzed as in B.

separated under non-reducing conditions. The gel was then reduced and submitted to a separating slab gel gradient. Silver-staining of the slab gel clearly revealed that subunits 4 and 6 were linked during the first electrophoresis. Since Cys23 is the sole cysteine residue of subunit 6, the disulfide bridge between su4D54C and su6C23 indicates a close proximity of the hydrophilic loop of subunit 4 and the N-terminus of subunit 6. Despite the presence of light chain immunoglobulins migrating in the 25−30 kDa range, a 4−6 dimer and a 4−f dimer were also evidenced by two-dimensional SDS−PAGE of an ATP synthase immunoprecipitate isolated from a crude D54C mitochondrial extract cross-linked with APDP (Figure 5C).

DISCUSSION

Cysteines were inserted into a hydrophilic loop (positions 47, 49, 54, and 55) located between two postulated membrane-spanning segments. The incubation of intact mitochondria with maleimides having different permeability properties led to the conclusion that subunit 6 and the mutated subunits 4 of the yeast ATP synthase displayed accessible targets to hydrophobic maleimides such as DACM. Except when using

leaky mitochondria, DACM did not modify OSCP, which is a potential target located on the F_1 side. The highly hydrophilic maleimide AMDA efficiently protected su4D54C from MPB-labeling, thus showing that Cys54 is located in a hydrophilic environment. On the contrary, the absence of MPB-labeling of su4N47C and su4E55C (data not shown) is in favor of a location of these residues in a more hydrophobic environment. As a result, the predicted loop connecting the two membrane-spanning segments should expose only a few residues (at least Cys54) to the aqueous phase. We cannot exclude a slight difference between the structure of the cysteine-containing hydrophilic loop and the wild type loop. However, this alteration did not significantly disturb the overall structure of the enzyme, as oxidative phoshorylations were conserved. Thus, a modification of the orientation of subunit 4 was excluded, since its C-terminal part is involved in the binding of F_1 (*14*). As a consequence, data provided by cysteine residues should reflect the global structure of the wild type hydrophilic loop, and validate the existence of the two postulated membrane-spanning segments linked by a short hydrophilic loop located near the surface of the membrane.

Cysteine substitution mutants were exploited to identify neighboring subunits with heterobifunctional cross-linking reagents. These experiments were performed (i) with intact mitochondria, (ii) with crude mitochondrial Triton X-100 extracts containing the solubilized ATP synthase, and (iii) with the purified enzyme. Three reagents with different spacer arms were used. ASIB and APDP with maximum cross-linking distances of 18.8 and 21 Å, respectively (*41*) linked subunits f and 4 and subunits 4 and 6. The absence of cross-linking with APA-Br indicates that the sulfhydryl group of su4D54C and subunit f should be at least 9 Å apart. The three reagents linked su4D54C and subunit 6, thus indicating a close proximity of the two subunits. In addition, the disulfide bridge induced by oxidation revealed that Cys54 of subunit 4 and Cys23 of subunit 6 are in contact. As subunit 6 and su4D54C were cross-linked when using either intact mitochondria or Triton X-100 extracts, a close proximity of the two subunits in the same complex was likely but not a cross-link occurring between two subunits of two different complexes. The hydrophobic plot of the yeast subunit 6 predicts a hydrophilic N terminal part that is longer than that of the bovine counterpart (not shown), and a location of Cys23 at the beginning of the first membrane-spanning segment. As Cys23 of subunit 6 was slightly modified by 200 μM MPB in comparison with su4D54C, we propose a location of the former residue in a less hydrophilic environment (not shown). From these results we conclude that subunits 6 and 4 are in contact, like subunits a and b of the *E. coli* ATP synthase (*42*, *43*). Moreover, the disulfide bridge occurring between su4D54C and su6C23 indicates a nearly similar location of both sulfhydryl groups on the outer phase of the inner mitochondrial membrane. As a result, the N-terminal part of subunit 6 should be in the intermembrane compartment which leads to an inverse location to that of the prokaryotic counterpart subunit a (*44*).

[3] Specific ATPase activities were 49.3 ± 2.2 and 50.5 ± 0.3 (μmol of P_i min^{-1}) (mg of protein)$^{-1}$ in the absence of oligomycin and 29.5 ± 1.5 and 27.3 ± 2.5 (μmol of P_i min^{-1}) (mg of protein)$^{-1}$ in the presence of oligomycin for D273-10B/A/H/U- and PVY219-purified enzymes, respectively.

It is unclear why none of the three reagents used did not cross-link subunits 6 and 4 in a wild type context. The absence of an effect may be due to local steric constraints which prevent access and orientation of the cross-linking reagents with the sulfhydryl group. However, Cys23 of subunit 6 of the wild type strain was modified by the maleimides DACM and MPB. Another explanation may be the low reactivity of the sulfhydryl group towards the alkylating reagents (APA-Br and ASIB) and pyridyl disulfide thiol interchange reagent (APDP). This problem is under investigation.

Belogrudov et al. (*45*) reported that dissuccinimidyl tartrate (cross-linking distance of 6.4 Å) produced f-b cross-links in bovine ATP synthase preparations but not in submitochondrial particles. We also observed an f−4 cross-link but only in intact mitochondria and crude mitochondrial Triton X-100 extracts with cysteinyl reagents having arms longer than 9 Å. The specificity of the reagents could explain this discrepency. In addition, an alteration in the F_0 structure of the purified enzyme due to the purification procedure could lead to modifications of the relative locations of subunits, as we observed a low amount of APDP or ASIB induced f−4 cross-link (data not shown) and a spontaneous disulfide bridge between subunits 4 and 6 with the purified enzyme, while intact D54C mitochondria and crude D54C Triton X-100 extracts had the f−4 cross-link and no or little 4−6 disulfide bridge. These data support the idea of structural modifications of the complex during purification. For example, the yeast ATP synthase preparation displayed 10% of the full specific ATPase activity which can be recovered by exogenous phospholipid addition (*11*). In this regard (except for the unknown 50 kDa cross-linked product), it appeared that the crude Triton X-100 extracts [final detergent concentration of 0.375% (w/v)] displayed close cross-link results to those of intact mitochondria.

In the model of Duncan et al. (*46*) the *E. coli* b-subunits should function as a stator forming a rigid connection between the α-β subunits and the a-subunit outside a ring of rotating DCCD binding proteins. If the eukaryotic enzyme is similar, subunit 4 (subunit b) should be in contact with OSCP and F_1 (*3*) and subunit 6. No significant ATPase activity decrease in the D54C purified enzyme having either spontaneously[3] or induced 6−4 cross-link was measured. As a consequence, subunits 4 and 6 do not display large relative movements during catalysis.

ACKNOWLEDGMENT

We thank Dr. P. V. Graves for advice and discussions during the course of this work and Dr. Ray Cooke for his contribution to the editing of the manuscript.

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BI9714971