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Rapid report

Evidence of a subunit 4 (subunit b) dimer in favor of the proximity of ATP synthase complexes in yeast inner mitochondrial membrane

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Abstract

Yeast mitochondria having either the D54C or E55C mutations in subunit 4 (subunit b), which is a component of the ATP synthase stator, displayed a spontaneous disulfide bridge between two subunits 4. This dimer was not soluble upon Triton X-100 extraction either at concentrations which extract the yeast ATP synthase or at higher concentrations. Increasing detergent concentrations led to a lack of the oligomycin-sensitive ATPase activity, thus showing an uncoupling between the two sectors of the mutated enzymes due to the dissociation of the subunit 4 dimer from the mutant enzyme. There is only one subunit 4 (subunit b) per eukaryotic ATP synthase. As a consequence, the results are interpreted as the proximity of ATP synthase complexes within the inner mitochondrial membrane. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Yeast; Mitochondria; ATP synthase; Subunit 4; Cross-linking

The F_1F_0-ATP synthase is responsible for aerobic synthesis of ATP. It exhibits a tripartite structure consisting of a headpiece (catalytic sector), basepiece (membrane sector) and a connecting stalk. The sector F_1 containing the headpiece is a water-soluble unit retaining the ability to hydrolyze ATP when in a soluble form. F_0 is embedded in the membrane and is mainly composed of hydrophobic subunits forming a specific proton pathway. The connecting stalk is constituted of the components from 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 model for energy coupling by F_1F_0-ATP synthase that has gained the most general support is the binding change mechanism [3]. This concept has been strengthened by the establishment of the crystal structure of the major part of the mitochondrial F_1 [4]. The affinity change for the substrates and products at catalytic sites are most likely coupled to proton transport by the rotation of the γ subunit ([5–8] for review). The ATP synthase thus operates as a rotary motor with a rotor consisting in F_1 *Escherichia coli*, of subunits γ and ε [9]. 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

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Abbreviations: ASIB, 1-(*p*-azidosalicylamido)-4-(iodoacetamido)butane; EDTA, ethylenediamine tetra-acetic acid; F_0 and F_1, integral membrane and peripheral portions of ATP synthase; OSCP, oligomycin-sensitivity-conferring protein; SDS, sodium dodecyl sulfate

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pathway [6,10]. As a consequence, subunits α, β, δ, a and b should be components of the stator in *E. coli*.

Yeast subunit 4, which is homologous to the b-subunit of the beef heart enzyme, is a component of the stator [11]. A stoichiometry of one subunit b per complex has been determined in eukaryotic F_1F_0-ATP synthase [12,13]. Like its counterpart in *E. coli*, the eukaryotic b-subunit appears to be necessary for a tight coupling between proton flux and ATP synthesis [14–19]. Subunit 4 contains 209 amino acid residues and has a molecular mass of 23 250 Da [20]. The structure of the eukaryotic b-subunit is composed of a predominantly hydrophobic N-terminal part, and a charged hydrophilic C-terminal part. The subunit is considered to traverse the membrane twice via two hydrophobic stretches of amino acids, with the N- and C-terminal parts emerging from the membrane on the F_1 side [13]. Recently, we reported that a short hydrophilic loop (residues 46–56) connecting the two predicted transmembrane α-helices was accessible to hydrophilic maleimide reagents from the intermembrane space [21]. In the present paper, we report the existence of a spontaneous disulfide bridge between two subunits 4 in two cysteine mutants.

The *Saccharomyces cerevisiae* strain D273-10B/A/H/U (MATα, *met6*, *ura3*, *his3*) [18] was the wild-type strain. The mutants in subunit 4 were named as (wild-type residue) (residue number) (mutant residue) where the residues were given a single-letter code. The strain containing the chromosomal copy of the ATP4 gene encoding the subunit 4D54C was obtained by homologous recombination of the PVY10 (MATα, *met6*, *ura3*, *his3*, ATP4::URA3) [18] strain with the 1580-bp *Eco*RI–*Sal*I DNA fragment containing the altered ATP4 gene. The mutant strain K7C was obtained by complementation of the PVY10 strain with the low copy vector pDR1 [22] bearing the mutated ATP4 gene. The mutant strain had generation time and mitochondrial ATPase activities similar to those of the wild-type.

Preparation of mitochondria and cross-linking experiments were according to [21]. The subunit 4 dimer was prepared from 2 mg of mitochondrial protein previously incubated with Cu(II)(1–10-phenanthroline)$_3$ for 1 h at 4°C. The reaction was stopped by addition of 10 mM EDTA. After centrifugation for 10 min at 10 000×*g*, the mitochondrial pellet was suspended in 0.2 ml of ATP synthase extraction buffer [21]. A 0.2-ml amount of Triton X-100 8% (w/v) was then added. After 20 min at 4°C, 0.4 ml of water was added and the mixture was centrifuged at 100 000×*g* for 15 min. The pellet was resuspended in water and centrifuged in the same conditions. The final pellet was resuspended in 0.1 ml of water, added to 1 ml of chloroform/methanol (1:1) and incubated for 2 h at room temperature under stirring. The mixture was centrifuged (10 000×*g*, 10 min) and the pellet was extracted for 2 h by 1 ml of chloroform/methanol (1:1) containing 0.5 M pyridinium acetate pH 7.0 [23]. The mixture was again centrifuged (10 000×*g*, 10 min) and the supernatant was dried under nitrogen and analyzed by SDS-PAGE.

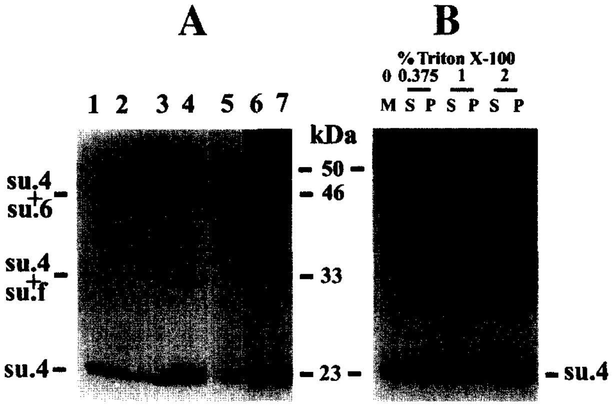

Fig. 1. A 50-kDa band reacted with antibodies raised against subunit 4 in D54C and E55C mitochondria. (A) Mitochondria (lanes 1–5) and purified D54C ATP synthase were incubated or not in the presence of 200 μM ASIB [21]. After irradiation at 365 nm, samples were dissociated and aliquots (30 μg protein) were submitted to Western blot analysis. Lane 1, wild-type; lane 2, wild-type+ASIB; lane 3, D54C; lane 4, D54C+ASIB; lane 5, E55C. D54C ATP synthase without (lane 6) and with ASIB (lane 7). (B) D54C mitochondria were incubated with increasing concentrations of Triton X-100. The extract was spun and pellets and supernatants were analyzed. M, mitochondria; S, supernatant; P, pellet; su., subunit. The blots were incubated with polyclonal antibodies raised against subunit 4 and used with a 1:10 000 dilution.

In a previous paper, we examined the topology of the yeast F_0 by using cysteine substitution mutants in subunit 4. Introduction of cysteinyl residues in the loop connecting two hydrophobic stretches of amino acids in the N-terminal part of the subunit showed the accessibility of the Cys-54 to hydrophilic maleimide reagents and the proximity of subunits 4 and 6, as demonstrated by the disulfide bridge occurring between su4D54C and su6C23 [21].

Western blot analysis of wild-type and D54C mitochondria revealed subunit 4 with the molecular mass of 23 kDa. In the absence of any cross-linking reagent, D54C and E55C mitochondria showed a new band migrating as a protein with a mass close to 50 kDa (Fig. 1A, lanes 3 and 5). On the other hand, the 50 kDa band was absent in N47C and L49C mitochondria (not shown) and also in wild-type mitochondria, whose subunit 4 is devoid of cysteine residue [20]. In addition, the 50 kDa band was not found in the D54C purified ATP synthase (Fig. 1A, lanes 6 and 7). Further analyses showed that the 50 kDa band was not detected in Triton X-100 extracts, since an increase in detergent concentration did not result in solubilization of the protein, which was always found in the pellet after ultracentrifugation (Fig. 1B). Other detergents such as cholate, lubrol, lauryl maltoside, Tween 20 and Nonidet P40 gave similar results (data not shown). As shown in Fig. 2A, the intensity of the 50 kDa band was increased upon oxidation, and the band disappeared upon reduction with 2% β-mercaptoethanol. In addition, it was insensitive to a 50 mM NEM incubation prior to dissociation (not shown), thus showing the existence in intact mitochondria of a disulfide bridge between subunit 4 and another subunit having a molecular mass of 20–25 kDa. The only subunits of yeast ATP synthase having both this mass and a cysteinyl group are OSCP and subunit 6, but the 50 kDa band did not react with antibodies raised against these proteins (not shown). On the other hand, a subunit 4 dimer linked by a disulfide bridge via Cys-54 was possible. To confirm this, D54C mitochondria were extracted according to a procedure previously used in our laboratory for quantitative extraction of subunit 4 [23]. Mutant mitochondria were incubated with $CuCl_2$ in order to increase the linkage amount, then the crude extract was submitted to a two-dimensional SDS-PAGE. Silver staining of the slab gel revealed only one spot, which is in favor of a dimer of subunit (Fig. 2B).

Table 1
Stoichiometry of cysteine-containing subunits of yeast ATP synthase

Subunit	Radioactivity (arbitrary units)	Ratio	Cysteine per molecule
α	1 646 859	2.9	1
γ	870 334	1.5	1
δ	448 445	0.78	1
4K7C	494 202	0.86	1
6	611 488	1.06	1
OSCP	532,063	0.92	1

The purification of the yeast ATP synthase has been described [26]. The cysteine-containing subunits of K7C ATP synthase were labeled by iodo[2-^{14}C]acetic (1.95 GBq/mmol) under denaturating conditions according to [13]. The subunits were separated by SDS-PAGE and the dried slab gel was exposed to an image plate scanner.

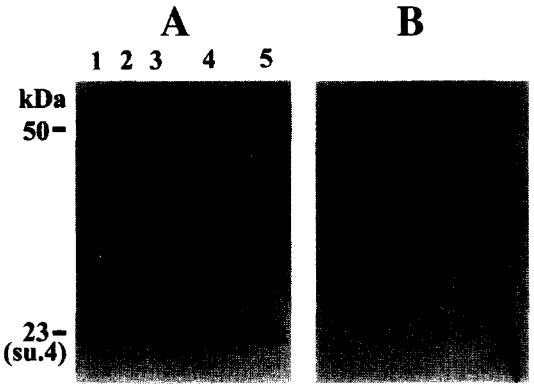

Fig. 2. Disulfide bridge between two subunits 4. (A) Lane 1, D54C mitochondria (30 μg); lanes 2–5, Triton X-100 ultracentrifugation pellets of D54C mitochondria incubated without (lanes 2 and 4) or with 1.5 mM $CuCl_2$ (lanes 3 and 5). The samples of lanes 4 and 5 were reduced by β-mercaptoethanol before electrophoresis. After transfer the blot was incubated with anti-subunit 4. (B) Two-dimensional gel electrophoresis of a crude organic extract of D54C mitochondria incubated with 1.5 mM $CuCl_2$. The first electrophoresis was run on a rod gel (12×0.35 cm inner diameter) containing a 15% running gel and a 7.5% stacking without β-mercaptoethanol treatment. Second dimension, 15% SDS-PAGE. The second dimension was done after incubation of the rod gel with 2% β-mercaptoethanol. The slab gel was silverstained [28]. su., subunit.

These results could be interpreted by the existence of a dimer of subunit 4 in the yeast ATP synthase, so the stoichiometry of the cysteine-containing subunits of mutant yeast ATP synthase was measured. K7C ATP synthase whose mitochondria, like N47C and L49C mitochondria do not display the dimer formation (unpublished result), was purified. Radiochemical labeling of cysteinyl groups with iodo[2-^{14}C]acetic acid has shown that there is one subunit 4 per yeast ATP synthase (Table 1). This result is in agreement with those of Collinson et al. [12,13] who established

Table 2
ATPase activities of yeast mitochondria and Triton X-100 extracts

Sample	ATPase activity		Remaining activity (%)
	−Oligomycin (μmol Pi/min/mg protein)	+Oligomycin (μmol Pi/min/mg protein)	
Wild-type			
None (mitochondria)	5.7 ± 0.2	0.5 ± 0.1	9
Triton X-100			
0.375%	6.8 ± 0.1	0.3 ± 0.1	4
1%	5.8 ± 0.1	0.3 ± 0.1	5
2%	5.9 ± 0.1	2.3 ± 0.1	39
D54C-mutant			
None (mitochondria)	5.2 ± 0.1	0.9 ± 0.1	17
Triton X-100			
0.375%	8.4 ± 0.2	2.4 ± 0.1	28
1%	7.4 ± 0.4	2.6 ± 0.1	35
2%	6.9 ± 0.2	4.4 ± 0.3	64

Wild-type and D54C mitochondria (0.9 mg) were extracted with Triton X-100 in a final volume of 0.18 ml. After ultracentrifugation (100 000 × *g*, 15 min), aliquots were taken to determine protein amount and ATPase activity. The ATPase activities were measured at pH 8.4 [27]. The activity found in the supernatant is presented. The specific activity was calculated from this data and from the protein amount. Assays were performed at 30°C with addition of 6 μg oligomycin per ml where indicated. Values reported are from three measurements with standard deviation.

a stoichiometry of one b-subunit in a purified beef heart ATP synthase complex which was fully active after reconstitution into unilamellar phospholipid vesicles [24]. An over-expression of subunit 4 in D54C and E55C mitochondria was unlikely as the 50-kDa band exists either in a strain bearing a low copy vector or in another containing a chromosomal copy of the mutated gene at its own locus. It may be therefore that ATP synthases in the inner mitochondrial membrane interact with their subunit 4. The absence of a dimer of subunit 4 in the D54C Triton X-100 extract and in the purified D54C ATP synthase is likely due to a destabilization of the dimer which was removed from the ATP synthase complexes having these disulfide bridges. As subunit 4 is an hydrophobic protein, the dimer is not soluble in detergents other than SDS, so it was recovered in the pellet after ultracentrifugation.

Removing subunit 4, which is a main component of the ATP synthase stator, must affect the activity of the complex, as it should disconnect the catalytic and the membrane sectors, as did disruption of the ATP4 gene [23]. Table 2 shows ATPase activities of both mitochondria and Triton X-100 extracts. Solubilization of ATP synthase complexes was nearly maximal at 0.375% Triton X-100 (w/v). Higher detergent concentrations resulted in a decrease in the specific ATPase activities and also in an alteration of the coupling of both ATP synthases, but in the presence of high Triton X-100 concentrations the oligomycin-sensitive ATPase activity of D54C extracts was more significantly decreased than that of the wild-type, thus showing an uncoupling between the catalytic and the membranous sectors. The decrease in oligomycin-sensitive ATPase activity is most probably the consequence of removing subunit 4 and not an effect of the mutation itself, because cysteine-54 is located on the outer phase of the inner mitochondrial membrane [21].

By using cross-linkers with different chemical specificities and different lengths, Belogrudov et al. [25] have reported the existence of a subunit b dimer in beef heart F_1-deficient submitochondrial particles, thus suggesting a stoichiometry of two b-subunits per complex. Our results could resolve the discrepancy concerning the stoichiometry of the eukaryotic b-subunit by making the hypothesis that ATP synthase complexes in yeast inner mitochondrial membrane are in contact, at least, at the short hydrophilic loop (residues 54 and 55) level. In addition, this interpretation indicates that ATP synthases are not structurally equivalent in yeast mitochondria because only some D54C and E55C complexes were spontaneously cross-linked.

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