FEBS Letters 580 (2006) 3427–3432

Characterization of dimeric ATP synthase and cristae membrane ultrastructure from *Saccharomyces* and *Polytomella* mitochondria

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Received 8 March 2006; revised 20 April 2006; accepted 21 April 2006

Available online 12 May 2006

Edited by Richard Cogdell

Abstract **There is increasing evidence now that F_1F_0 ATP synthase is arranged in dimers in the inner mitochondrial membrane of several organisms. The dimers are also considered to be the building blocks of oligomers. It was recently found that the monomers in beef and the alga *Polytomella* ATP synthase dimer make an angle of ~40° and ~70°, respectively. This arrangement is considered to induce a strong local bending of the membrane. To further understand the packing of dimers into oligomers we performed an electron microscopy analysis of ATP synthase dimers purified from *Saccharomyces cerevisiae*. Two types of dimers were found in which the angle between the monomers is either ~90° or ~35°. According to our interpretation, the wide-angle dimers (70–90°) are "true-dimers" whereas the small-angle dimers (35–40°) rather are "pseudo-dimers", which represent breakdown products of two adjacent true dimers in the oligomer. Ultrathin sectioning of intact *Polytomella* mitochondria indicates that the inner mitochondrial or cristae membrane is folded into lamellae and tubuli. Oligomers of ATP synthase can arrange in a helical fashion in tubular-shaped cristae membranes. These results strongly support the hypothesized role of ATP synthase oligomers in structural determination of the mitochondrial inner membrane.**

Keywords: ATP synthase; Dimer; Electron microscopy; Mitochondria; *Saccharomyces cerevisiae*; *Polytomella*

1. Introduction

The proton F_1F_0 ATP synthase is a ubiquitous enzyme that is found in virtually all living organisms. It is a multi-subunit complex of about 600 kDa involved in rotary catalysis. In prokaryotes the enzyme consists of an F_0 intrinsic membrane domain with three different subunits named a, b and c and an extramembranous F_1 domain (or headpiece), including a central stalk, with five different subunits α, β, γ, δ and ε. Three α and β subunits shape the F_1 headpiece; the γ, and ε are present in single copies in the central stalk. Most of the F_0 domain is formed by a multimer of 10 c subunits. The c-subunit multimer of the F_0 domain and the $\alpha_3\beta_3$ F_1 headpiece are linked by the central stalk and additionally by a peripheral stalk composed of two b subunits. The peripheral stalk is connected to the top of the $\alpha_3\beta_3$ F_1 headpiece via the δ subunit. During catalysis a proton membrane gradient drives the rotation of the ring of multiple copies of the c subunit [1]. This results in a clockwise rotation of the γ subunit of the F_1-ATPase and in the synthesis of ATP.

Mitochondrial F_1F_0 ATP synthases contain the same eight subunits, though some have a different name such as the OSCP subunit, which is the counterpart of the prokaryotic δ subunit. There are at least eight small subunits in addition to those of the prokaryotic enzyme [2]. In *Saccharomyces cerevisiae* there is one additional subunit located in the central stalk (the mitochondrial specific ε subunit). Most of the remaining ones are located in the F_0 domain (d, e, f, g, h, i and k) where they are positioned close to the peripheral stalk. Atomic resolution structures have revealed the folding of the α, β, γ, δ and ε subunits of the F_1 domain [3,4] and based on biochemical studies there is a recent lower resolution model for the arrangement of the four subunits consisting the peripheral stalk or stator in beef [5]. There is, however, no detailed structural information of the many (small) subunits of bovine or yeast ATP synthase, which form the F_0 domain outside the subunit c multimer.

Although the proton ATP synthase is catalytically active in its monomeric form a dimeric ATP synthase supercomplex from yeast mitochondria has been characterized by BN/SDS PAGE [6] and other techniques. This supercomplex includes the above-mentioned small subunits f, i, e, g, and k, the latter three of which are thought to be involved in dimer formation. Recent data show that yeast cells deficient in the dimer-specific subunits e or g lack dimeric ATP synthase [7,8]. Some data also point to an involvement of one of the subunits of the peripheral stalk, subunit b, in dimer formation [9]. Similar dimeric ATP synthase supercomplexes were found in a wide range of organisms such as beef [10], *Arabidopsis* [11], *Chlamydomonas* [12] and *Polytomella* [13,14].

Several biochemical studies indicate in addition to the dimeric conformation a further packing of ATP synthase into oligomers. The oligomerization of the ATP synthase has been proposed to determine mitochondrial morphogenesis [7,8,14]. Yeast mutants that lack the ATP synthase subunits e or g show a different type of folding of the inner mitochondrial or cristae membrane. This membrane is heavily folded, but mutants show an onion-like packing of cristae membranes. A GTPase named Mgm1p serves as a regulator of subunit e stability, ATP synthase assembly and cristae morphology [15]. The only

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0014-5793/$32.00
doi:10.1016/j.febslet.2006.04.097

direct evidence for ATP synthase oligomers was obtained in an electron microscopy study by using rapid-frozen deep-etched mitochondria from the unicellular freshwater organism *Paramecium* [16]. One of the best ways to study subcellular structures by electron microscopy is to embed them without chemical fixation in amorphous ice and to perform a three-dimensional reconstruction by electron tomography [17,18]. But for relatively large objects like mitochondria it remains particularly difficult to obtain a resolution sufficient to see the packing of individual copies of the major protein complexes like the ATP synthases, because the attainable resolution is directly related the size of the object [19]. In the best current available reconstructions the F_1 headpieces come into focus, but they do not show how the oxidative phosphorylation complexes are precisely organized [17].

Until now a low-resolution structural characterization of the ATP synthase dimer has been carried out in parallel for two different species: beef [20] and the colorless alga *Polytomella* [14]. In both cases it was found that the two F_1F_0 monomers are making an unexpected angle which was ~40° in the case of beef and ~70° for *Polytomella*. Because of this angle the membrane around the F_0 parts of the dimer is curved. It was concluded that this kink drives the mitochondrial cristae membrane to adopt a local curvature [14,20]. A major difference between the two dimers, however, is a difference of ~30° between the two angles. This discrepancy may be explained by the fact that the two species are not closely related and have a different subunit composition. But it may also be possible that the association of monomers in the dimers is different. To get further insight into the dimer configuration we performed single particle electron microscopy analysis on a third species, the yeast *S. cerevisiae*. Since *Polytomella* ATP synthase dimers were found to be unusually stable [14], additional ultrathin sectioning of these mitochondria was performed to investigate the overall membrane packing of the oxidative phosphorylation complexes. The analysis of the yeast dimers indicates that detergent solubilization of mitochondrial membranes leads actually to two distinct types of dimers, either with an angle of about 90° between the long axes of the F_1F_0 monomers or with an angle of 35°. Both types of dimers are considered to originate from an oligomeric organization of ATP synthases in rows. Ultrathin-sectioning and negative staining of osmotically shocked *Polytomella* mitochondria show that such oligomers are (partly) arranged in a helical fashion in tubular-shaped cristae membranes.

2. Materials and methods

2.1. Cultivation of *Polytomella spp. and* Saccharomyces cerevisiae

Polytomella spp. (198.80, E.G. Pringsheim) was obtained from the "Sammlung von Algenkulturen der Universität Göttingen" (Germany) and cultivated as described in Dudkina et al. [14]. *S. cerevisiae* (strain Y187) was cultivated in YPD medium. For mitochondrial isolations, cells were transferred into lactate medium (2.6 mM glucose, 7.3 mM KH_2PO_4, 18.7 mM NH_4Cl, 4.5 mM $CaCl_2$, 8.6 mM NaCl, 2.9 mM MgCl, 2.2% lactate).

2.2. Preparation of mitochondria

Mitochondria of *Polytomella* were isolated by differential centrifugation and Percoll density gradient ultracentrifugation as outlined previously [14]. Isolation of yeast mitochondria was based on differential centrifugations and sucrose gradient ultracentrifugation as described by Meissinger et al. [21]. Mitochondria were shock-frozen using liquid nitrogen and stored at −80 °C until use.

2.3. Purification of dimeric ATP synthase

The membrane-bound protein complexes of yeast and *Polytomella* were solubilized using digitonin solution (5% detergent, 30 mM HEPES, 150 mM K-acetate, 10% glycerine, pH 7.4) and separated by sucrose gradient ultracentrifugation (gradients of 0.3–1.5 M sucrose, 15 mM Tris base, pH 7.0, 20 mM KCl, 0.2% digitonin; centrifugation for 17 H at 150,000 × *g*/4 °C). Afterwards the gradients were fractionated and the protein complex compositions of the fractions analyzed by 1D Blue-native PAGE [22].

2.4. Electron microscopy

Selected fractions of the gradients including dimeric ATP synthase were directly used for electron microscopy and single particle analyses [23]. For ultrathin sectioning, *Polytomella* mitochondria were isolated according to [14], pelleted and double fixed with glutaraldehyde and osmium tetroxide. Fixed mitochondria were dehydrated with acetone and embedded in Epon. Ultrathin sections were made on an ultramicrotome and double stained with uranyl acetate and lead citrate.

For studying the localization of the oxidative phosphorylation system in the inner membrane before detergent disruption *Polytomella* mitochondria were osmotically shocked by diluting them 10× in distilled water on electron microscopy grids and directly stained with uranyl acetate.

3. Results

3.1. Purification of yeast ATP synthase dimers

Solubilization of isolated yeast mitochondria by digitonin and analysis of the solubilized protein complexes by 2D Blue native/SDS PAGE allowed to monitor the known complexes of the respiratory chain (Fig. 1). In accordance with previous publications [6], ATP synthase is present in two forms, a monomeric complex of about 500 kDa and a dimeric complex of about 1000 kDa. In-between, supercomplexes of dimeric complex III and one or two copies of monomeric complex IV are visible. Furthermore, two forms of dimeric complex III are present below the monomeric ATP synthase complex, which represent the intact dimer and a subcomplex of the dimer lacking the Rieske FeS subunit and another 8.5 kDa subunit. Both subunits are known to easily get detached from the dimeric complex III [24]. The ratio of monomeric and dimeric ATP synthase very much depends on the detergent used for solubilization [6]. The dimer is best stabilized in the presence of low Triton X-100 concentrations. However, digitonin and dodecylmaltoside also proved to be suitable detergents for ATP synthase supercomplex stabilization [10].

In contrast, ATP synthase of *Polytomella* is exclusively visible in the dimeric form on 2D Blue native/SDS gels independently of the type of non-ionic detergent used for solubilization (Fig. 1, [13,14,25]). The extraordinary stability of the dimer most likely is due to an additional large subunit called "mitochondrial ATP synthase associated protein" (MASAP), which runs at 60 kDa on the second gel dimension (Fig. 1).

For successive EM analysis of yeast ATP synthase, Blue-native PAGE was substituted by sucrose density gradient ultracentrifugation to obtain protein complexes in solution. The gradients were fractionated and small aliquots of the fractions were analyzed by Blue-native PAGE to monitor their protein complex composition. A fraction close to the bottom of the gradients (fraction 5) included pure ATP synthase dimers. The corresponding fractions were directly used for EM and single particle analysis.