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Distances between the b-subunits in the tether domain of F_0F_1-ATP synthase from *E. coli*

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Abstract

The arrangement of the b-subunits in the holo-enzyme F_0F_1-ATP synthase from *E. coli* is investigated by site-directed mutagenesis spin-label EPR. F_0F_1-ATP synthases couple proton translocation with the synthesis of ATP from ADP and phosphate. The hydrophilic F_1-part and the hydrophobic membrane-integrated F_0-part are connected by a central and a peripheral stalk. The peripheral stalk consists of two b-subunits. Cysteine mutations are introduced in the tether domain of the b-subunit at b-40, b-51, b-53, b-62 or b-64 and labeled with a nitroxide spin label. Conventional (9 GHz), high-field (95 GHz) and pulsed EPR spectroscopy reveal: All residues are in a relatively polar environment, with mobilities consistent with helix sites. The distance between the spin labels at each b-subunit is 2.9 nm in each mutant, revealing a parallel arrangement of the two helices. They can be in-register but separated by a large distance (1.9 nm), or at close contact and displaced along the helix axes by maximally 2.7 nm, which excludes an in-register coiled-coil model suggested previously for the b-subunit. Binding of the non-hydrolysable nucleotide AMPPNP to the spin-labeled enzyme had no significant influence on the distances compared to that in the absence of nucleotides.

Keywords: H^+-ATP synthase; b-subunit; Site-directed spin labeling; MTSL; EPR spectroscopy; DEER

1. Introduction

F_0F_1-ATP synthases catalyze the proton transport-coupled synthesis of ATP from ADP and phosphate in bacteria, chloroplasts and mitochondria [1]. The enzyme consists of two large domains: a membrane-integrated F_0-part catalyzing the transmembrane proton transport and a hydrophilic F_1-part containing the nucleotide and phosphate binding sites. In the F_0F_1-ATP synthase from *E. coli*, the F_1-part has a subunit composition of $\alpha_3\beta_3\gamma\delta\varepsilon$ (see Fig. 1a). The F_0-part consists of ab_2c_{10-14} [2] and the c-subunits form a ring in the membrane [3].

According to the 'binding change' theory, the three catalytic nucleotide binding sites of the β-subunits undergo conformational changes, adopting in subsequent steps the 'open', 'tight' and 'loose' conformation. This is accomplished by sequential "docking–undocking" steps of the γ-subunit to the three different αβ-pairs, i.e. by a rotation of the γ-subunit which synchronizes the catalytic reaction at the three catalytic sites [4]. The high resolution X-ray structure supported this mechanism [5] and rotation of the γ-subunit during ATP hydrolysis was demonstrated [6,7] and visualized by video-microscopy [8].

With respect to proton transport-coupled ATP synthesis in the holo-enzyme, it was suggested that the γ-subunit is connected with the ring of c-subunits and that proton transport through the enzyme is accomplished by rotation of the c-ring in the membrane. As the c-ring

Abbreviations: AMPPNP, adenosine-5′(β,γ-imido) triphosphate; DEER, double electron–electron resonance; EPR, electron paramagnetic resonance; MTSL, (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) methanethiosulfonate; TMR-M, tetramethylrhodamine-5-maleimide

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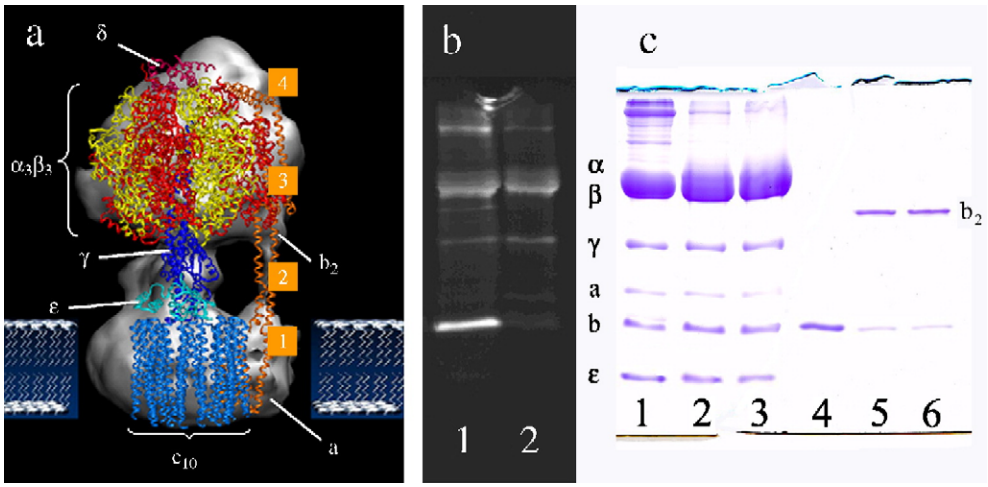

Fig. 1. (a) Membrane-embedded F_0F_1-ATP synthase from *E. coli* derived from homology modeling [17] and electron microscopy [18]. According to [23], the b-subunit (orange) can be divided into the transmembrane domain (pictured as <1>), tether domain <2>, dimerization domain <3> and δ-binding domain <4>. Each b-subunit was labeled with the nitroxide spin-label MTSL at residues 40, 51, 53, 62 and 64 which are located in the tether or dimerization domain, respectively. (b) Fluorograms of electrophoretic separation (SDS-PAGE) of the subunits of F_0F_1-ATP synthase. Lane 1: F_0F_1 labeled with TMR-M. Mainly, the b-subunits are labeled. Subunits α, β and γ show weak fluorescence intensities. Other subunits are not labeled. Lane 2: Spin-labeled F_0F_1 was labeled with TMR-M. Subunit b shows very weak fluorescence intensity; the intensities of other subunits are comparable to lane 1. (c) SDS-PAGE of cross-linking experiment. Lane 1: F_0F_1 without cross-linking treatment. Subunit c ran out of the gel; the δ-subunit can be seen as a weak band above the b-subunit. Lanes 2 and 3: F_0F_1 treated with 50 μM $CuCl_2$. The reaction was stopped by the addition of 2 mM EDTA after 30 min and 120 min, respectively. No b-dimer can be seen. Lane 4: truncated b-subunit b_{34-156}T62C (without residues 1 to 33). Lane 5 and 6: b_{34-156}T62C treated with 50 μM $CuCl_2$. The reaction was stopped by adding 2 mM EDTA after 30 min and 120 min, respectively, indicating cross-linking (>90% of b-dimer).

interacts with the γ- and ε-subunits, this drives the rotation of the γ-subunit in the F_1-part. Correspondingly, the $\gamma\varepsilon c_{10\text{-}14}$ complex is called "rotor", $\alpha_3\beta_3\delta ab_2$ the "stator" [9–12].

Quantitative models describing the generation of the required power for ATP synthesis have been presented [13–15]. Recently, it has been shown with single molecule spectroscopy that during proton transport-driven ATP synthesis, the γ-subunit rotates relative to the stator subunits and that the direction of rotation is reversed during ATP hydrolysis [16,17] (a model of F_0F_1-ATP synthase is shown in Fig. 1a combining data from electron microscopy [18] and a homology model [17]).

The movement of the rotor part in the enzyme requires a firm connection between the stator subunits. According to the current structural models, the two b-subunits connect the membrane-integrated stator part (a-subunit) with the hydrophilic stator part ($\alpha_3\beta_3\delta$-subunits). Electron microscopy showed that F_1 and F_0 are linked by a central (γ- and ε-subunit) and a peripheral (b-subunits) second stalk [18–20]. A high-resolution X-ray structure of the b-subunits in the holo-enzyme is not available yet, however some data were published on the structure of parts of the subunit and its interaction with the F_1-part [21,22]. The b-subunit can be divided into 4 major parts: the N-terminal transmembrane domain (see domain <1> in Fig. 1a, residues 1 to 22), followed by the tether domain (<2>, residues 23 to 52), the dimerization domain (<3>, residues 53 to 122) [23] and the δ-binding domain (<4>, residues 123 to 156) [24]. The structure of an isolated fragment of the b-subunit comprising residues 1 to 34 has been solved by NMR, revealing a hydrophobic membrane-spanning α-helix [21]. Recently, the binding strength of the b-subunits to the F_1-part was determined [25,26]. Further biochemical approaches such as circular dichroism spectroscopy, chemical cross-linking, analytical ultracentrifugation and deletion analysis revealed that the structure of the peripheral stalk consists of a highly extended conformation containing ~80% α-helix [27–34].

Structural information about the b-subunit in the holo-enzyme is needed to understand the role of the b-subunit in the stator part of the enzyme. The tether domain was used for fluorescence labeling in a recent single-molecule investigation of proton transport-coupled ATP synthesis [17], however very little is known about its structure.

We used a site-directed mutagenesis spin labeling EPR approach to investigate the structure of the b-subunits in the holo-enzyme. Previous spin-label EPR studies have targeted the β-subunit in F_1-ATPase using native cysteines [35] and mutagenesis to introduce spin labels in the isolated soluble b-subunit of ATPase [36,37].

In the present work, a series of mutants along a stretch extending from the tether domain to the dimerization domain of the b-subunit was prepared. As there are two b-subunits in the enzyme, mutagenesis to replace one codon of the b-gene by the cysteine-codon results in the substitution of two cysteines in the holo-enzyme. The mutants prepared were b-I40C, b-H51C, b-D53C, b-T62C and b-Q64C. In each case, the holo-enzyme was spin labeled with the nitroxide label MTSL, resulting in the spin-labeled mutants F_0F_1-bI40C, F_0F_1-bH51C, F_0F_1-bD53C, F_0F_1-bT62C and F_0F_1-bQ64C.