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De-novo modeling and ESR validation of a cyanobacterial F_oF_1–ATP synthase subunit *bb′* left-handed coiled coil ☆

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ABSTRACT

The structure and functional role of the dimeric external stalk of F_oF_1–ATP synthases have been very actively researched over the last years. To understand the function, detailed knowledge of the structure and protein packing interactions in the dimer is required. In this paper we describe the application of structural prediction and molecular modeling approaches to elucidate the structural packing interaction of the cyanobacterial ATP synthase external stalk. In addition we present biophysical evidence derived from ESR spectroscopy and site directed spin labeling of stalk proteins that supports the proposed structural model. The use of the heterodimeric *bb′* dimer from a cyanobacterial ATP synthase (*Synechocystis* sp. PCC 6803) allowed, by specific introduction of spin labels along each individual subunit, the evaluation of the overall tertiary structure of the subunits by calculating inter-spin distances. At defined positions in both *b* and *b′* subunits, reporter groups were inserted to determine and confirm inter-subunit packing. The experiments showed that an approximately 100 residue long section of the cytoplasmic part of the *bb′*-dimer exists mostly as an elongated α-helix. The distant C-terminal end of the dimer, which is thought to interact with the δ-subunit, seemed to be disordered in experiments using soluble *bb′* proteins. A left-handed coiled coil packing of the dimer suggested from structure prediction studies and shown to be feasible in molecular modeling experiments was used together with the measured inter-spin distances of the inserted reporter groups determined in ESR experiments to support the hypothesis that a significant portion of the *bb′* structure exists as a left-handed coiled coil.

1. Introduction

F_oF_1–ATP synthases provide the bulk of ATP that is needed for metabolic and catabolic processes as well as for cellular and organismal movement [1–3]. The synthase is found in the energy coupling membranes of thylakoid membranes, the mitochondrial inner membranes and the plasma membranes of bacteria. The subunit stoichiometry of the membrane-embedded F_o sector was reported to be 10–15 copies of subunit *c* for synthases of different origin, one subunit *a*, and one or two subunits *b* that make up much of the external stalk linking F_o to the catalytically active F_1-ATPase. F_1 contains five conserved subunits arranged in an $\alpha_3\beta_3\gamma\delta\varepsilon$ complex. Eubacterial ATP synthases contain two identical subunits *b* while photosynthetic organisms contain heterodimeric *bb′* subunits (called subunits I and II in chloroplast ATP synthase). Mitochondrial ATP synthases contain only one subunit *b*, but other subunits (*d* and F_6) are present that stabilize the overall external stalk–stator structure (for recent reviews see [4–6]). Subunit δ (OSCP in mitochondrial enzymes) seems to facilitate and strengthen the link between the *b*-dimer and the F_1-part of the enzyme [7].

The F_o-sector enables proton translocation across the energy coupling membrane down an electrochemical gradient and couples, via rotation of the ring of subunits *c*, the energy of the proton gradient to the energy needed for ATP product release from F_1 during catalysis. The rotary motions within F_o and F_1 mesh with different step sizes. Rotation of the subunit *c* ring occurs with steps between 36° and 24° per translocated proton. The rotational steps of subunit γ were shown to be distinct 120° steps for one ATP synthesis or ATP hydrolysis event [8–10]. The mechanism of the gearing of these different rotational movements is not only of great significance to the overall mechanism of the ATP synthase, it is also of great interest from engineering points of view due to the extremely high thermodynamic efficiency of this rotary motor [11].

A major player that may link these rotations is the stator subunit *b* dimer, which connects the non-rotating F_o subunit *a* to the non-rotating F_1 subunits ($\alpha_3\beta_3$). The *bb* or *bb′* dimer may also closely contact the rotating *c*-subunit ring as well as the rotating ε-subunit of

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F_1. It seems plausible that elastic conformational changes of the *b*-dimer may be involved in coupling the unequal rotational steps of F_o- and F_1-subunits [12]. Alternatively, the elasticity may take the form of winding or unwinding of the motor subunit γ coiled coil structure itself and not require additional participation by the external stalk. Combinations of both alternatives may also occur. Testing these models requires knowledge of conformational changes of the *b*-dimer during catalytic turnover. The prerequisite for these studies is a detailed knowledge of the structure and dimer packing of the external stalk.

X-ray structural models of the external stalk of the mitochondrial subunit complex bdF_6 were reported that show close packing of the stalk onto the surface of F_1 [13–15]. That the bdF_6 complex crystallized in such a perfectly F_1-complementary structure suggests that the external stalk may function as a rigid stator into which little energy from rotational steps could be transferred and later recovered. This may imply that the energies of intermediate steps required for coupling stem more from elastic deformations of subunit γ during catalysis. Experiments using bacterial ATPases, however, showed an unprecedented degree of structural plasticity in the *b*-homodimer. Almost 10% of the overall length of the protein could be deleted or inserted while still maintaining function [16,17]. Even *b*-subunits of unequal length retained catalytic activity [18]. Parts of the *b*-dimer could be exchanged for *b*-sequences from different species without complete loss of activity, although it is worthwhile to note that the parts of *b* thought to interact directly with F_1 were not interchangeable [19]. Tight interaction of the *b*-dimer with F_1 had previously been mapped to the C-terminal half of the *Escherichia coli* stator [20].

Controversy still exists about the packing of homodimeric *bb* structure, and much remains to be learned about the heterodimeric *bb′* external stalks from photosynthetic organisms. An X-ray structural model of a monomeric *E. coli b*-subunit dimerization domain was presented by the Dunn laboratory [21]. Visual inspection of a putative dimerization interface led the authors to speculate that *b* forms a right handed dimeric coiled coil. Right-handed coiled coil helical packing has been predicted to occur when interface residues occur in undecad (11-residue) repeats, allowing close packing interactions of *a* and *h* positions, usually occupied by small amino acid side chains, and *d* and *e* positions that are more peripheral and accommodate larger side chains [22]. So far no right-handed *dimeric* coiled coil has been shown to stably exist although trimers and tetramers have been observed. The dimeric right handed coiled coil therefore remains hypothetical. The X-ray model of the *b*-monomer [21] did, however, trigger a series of cysteine cross-linking studies from Dunn et al. that have been interpreted as supporting an unusual staggered right-handed homodimeric coiled coil [23,24].

The experimental protocols reported in these cross-linking studies show that very long incubation times under oxidative conditions were employed (either 24 h of rigorous stirring in the presence of air and $CuCl_2$ [23] or 72 h of dialysis in $CuCl_2$ solutions [24]). Excess cysteine in the experiments was intended to prevent non-specific cross-links. It seems very likely that during the extended incubation times employed the protective cysteine would have oxidized to cystine. Lack of reversibility of the cross-linking reactions and the long reaction times would in our estimation lead to accumulation of cross-linked products that reflected good disulfide formation chemistry but not necessarily stable low energy protein conformations. Oxidation of cysteines might in fact be obtained only in rarely occupied protein conformational states that present optimal reaction orientations of the cysteines. For these reasons we believe interpretation of these results should be viewed with caution. The results of these studies were interpreted by Dunn et al. [23,24] to indicate cross-links between introduced cysteines with an off-set of 4, 7 or 11 amino acids. These offsets would represent approximately one, two or three helical turns, respectively, between similar interfacial positions. It should be noted that only an offset of 11-residues is consistent with an uninterrupted right-handed coiled coil in this region as also proposed by Dunn.

A different approach to elucidating the *b* dimer structure was performed recently by our group [25,26]. We used structure prediction programs that identified extensive heptad repeat sequences in *b*-subunits from a variety of ATP synthases. Heptad repeats are often indicative of the propensity to form left-handed coiled coils. Molecular modeling strongly suggested that the *E. coli* subunit *b* dimer could fold into left-handed coiled coil structures [26]. These left-handed coiled coil models could be superposed with the monomeric subunit *b* X-ray structural model from Dunn [21], supporting further the validity of the left-handed coiled coil model. Experimental support for the left-handed coiled coil model was obtained by the excellent fit of 38 inter-subunit distance restraints that were obtained after site-specific spin-labeling of introduced cysteine residues and from ESR spectroscopy analyses [25].

In this report we describe enhanced efforts to obtain structural information about the external stalk of F_1F_o–ATP synthases. In these new studies we used a heterodimeric *bb′* from a cyanobacterial ATP synthase. This allowed us to acquire both intra- and intermolecular ESR-derived distance data since *b* and *b′* are encoded by different genes and allowed insertion of reporter groups either within one of the dimers or in each of the subunits independently. Structure prediction and improved modeling techniques emphasizing classic knobs in holes packing predicted by Crick [27] strongly suggested that the cyanobacterial *bb′* dimer can form, similar to the homologous *E. coli b*-homodimer, a stable, left handed coiled coil structure. Experimentally determined intra- and inter-chain distances agreed with distances obtained in molecular models of the spin-labeled proteins. These results strongly support the hypothesis that the external *bb′* stalk can adopt a left-handed, unstaggered coiled coil structure.

2. Materials and methods

2.1. Expression plasmids

The polar domains of subunit *b* (residues 49–179) and *b′* (residues 30–143) from the cyanobacterium *Synechocystis* sp. PCC 6803 ATP synthase were expressed using the plasmids pETb_{sol} and pETb'_{sol} [28]. Both subunits contained N-terminal His-6 or His-10 tags for easier purification. Single cysteine mutations were introduced using the Quikchange™ mutagenesis kit (Stratagene). The success of the mutation reactions was verified by DNA sequencing.

2.2. Protein expression

The wild-type and mutant polar domains of the subunits *b* and *b′* were expressed individually in *E. coli* strain BL21 (DE3) (Stratagene). The cells were grown in LB medium at 37 °C and constant shaking of 300 rpm. The expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at a culture density OD_{600} of 0.5 to 0.8. The cells were harvested at OD_{600} of 2–3 and were stored as wet pellets at −80 °C.

2.3. Protein purification

The cell pellets (5–10 g) were quick-thawed at 37 °C and were then incubated on ice in 50 mM Tris–HCl, pH 8.2 (5 ml per 1 g of wet pellet) that was supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF). The cells were broken through repeated passage through an EmulsiFlex-C5 homogenizer (Avestin, Ottawa, Canada). All purification steps thereafter were carried out at 1–4 °C. Cell debris was removed by slow speed centrifugation at 46,000 ×*g* for 30 min and finally bacterial membranes were pelleted by centrifugation at 340,000–360,000 ×*g* for 1 h. The resulting supernatant was decanted and supplemented with 30 mM imidazole, 100 mM NaCl and 1 mM dithiothreitol (DTT). The solution was