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3D reconstruction and flexibility of the hybrid engine *Acetobacterium woodii* F-ATP synthase

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Abbreviations: EM, Electron microscopy; RMSF; root mean square fluctuation

Abstract

The Na⁺-translocating F₁F₀ ATP synthase from *Acetobacterium woodii* (AwF-ATP synthase) with a subunit stoichiometry of $\alpha_3:\beta_3:\gamma:\delta:\epsilon:a:b_2:(c_{2/3})_9:c_1$ represents an evolutionary path between ATP-synthases and vacuolar ATPases, by containing a heteromeric rotor *c*-ring, composed of subunits *c*₁, *c*₂ and *c*₃, and an extra loop ($\gamma_{195-211}$) within the rotary γ subunit. Here, the recombinant AwF-ATP synthase was subjected to negative stain electron microscopy and single particle analysis. The reference free 2D class averages revealed high flexibility of the enzyme, wherein the F₁ and F₀ domains distinctively bended to adopt multiple conformations. Moreover, both the F₁ and F₀ domains tilted relative to each other to a maximum extent of 28° and 30°, respectively. The first 3D reconstruction of the AwF-ATP synthase was determined which accommodates well the modelled structure of the AwF-ATP synthase as well as the $\gamma_{195-211}$ -loop. Molecular simulations of the enzyme underlined the bending features and flexibility observed in the electron micrographs, and enabled assessment of the dynamics of the extra $\gamma_{195-211}$ -loop.

1. Introduction

In bacteria, ATP synthesis is mainly catalyzed by the F_1F_0 ATP synthase during the process of oxidative phosphorylation, which uses the electrochemical H^+ - or Na^+ -potential to drive the synthesis of ATP from ADP and inorganic phosphate [1]. In the anaerobic acetogenic bacterium *Acetobacterium woodii*, which is considered to have one of the oldest metabolic pathways on Earth, carbon fixation is coupled to the synthesis of ATP via an ancient respiratory system consisting of a Na^+ -translocating ferredoxin:NAD oxidoreductase (Rnf) and a Na^+ -ATP synthase. The Na^+ -dependent F_1F_0 ATP synthase (AwF-ATP synthase) of *A. woodii* is comprised of a water soluble F_1 part, containing the subunits $\alpha_3:\beta_3:\gamma:\delta:\epsilon$, and a membrane embedded F_0 part, including the subunits *a*, *b* and *c* [2-4]. The rotating central stalk subunits $\gamma\epsilon$ and the peripheral stalk subunits $b_2\delta$ connect the catalytic $\alpha_3:\beta_3$ domain and the Na^+ -translocation ac_{10} ensemble [5]. The *atp* operon of *A. woodii* is unique, since it contains three genes, *atpE*₁, *atpE*₂ and *atpE*₃ that code for the three *c* subunits *c*₁, *c*₂ and *c*₃, respectively, with a stoichiometry of 1:8.3:0.7 [6-8]. The *c*₂ and *c*₃ are two identical bacterial F_0 -like *c* subunits, while *c*₁ resembles the *c*-subunit of eukaryotic V_1V_0 -ATPases [9]. Subunits *c*₁ and *c*₂ are essential, as demonstrated by the diminished ATP hydrolysis activity and Na^+ -translocation of the Δc_1 or Δc_2 deletion mutants [8].

Most recently, we described the first 2D projections of the recombinant AwF-ATP synthase derived from negative stain electron microscopy (EM) and the unique extra loop in subunit γ , formed by amino acids 195-211 ($\gamma_{195-211}$; $^{195}\text{TSGKVKTTEETKEEKSK}_{211}$) [5]. A generated $\gamma_{\Delta 195-211}$ AwF-ATP synthase deletion mutant lost its capacity for ATP formation and Na^+ -transport when reconstituted into proteoliposomes, reflecting a role for this extra $\gamma_{195-211}$ -loop [5] in the dynamic process of coupling of Na^+ -translocation in the ac_{10} ensemble and ATP synthesis in the $\alpha_3:\beta_3$ headpiece.

Here, we extended these structural and mechanistic studies by presenting the first 3D reconstruction of the *AwF*-ATP synthase using negative stain EM. The 3D EM map revealed that the subunits $\alpha_3:\beta_3:\gamma:\delta:\epsilon:a:b_2:(c_{2/3})_9:c_1$ are accommodated well within the map, including the $\gamma_{195-211}$ -loop. Single particle analysis of the enzyme highlighted the flexibility of the F_1 - and F_0 parts relative to each other, which was confirmed by molecular simulations and highlight the dynamic features of the $\gamma_{195-211}$ -loop.

2. Material and Methods

2.1. Cloning, protein expression and purification

Cloning, expression and affinity purification of the *AwF*-ATP synthase was performed as previously described [5]. *E. coli* DK8 cells containing the plasmid encoding the enzyme was cultivated in 2xYT-medium at 37 °C under aeration. To solubilize the *AwF*-ATP synthase, the membrane pellet, which was prepared by ultracentrifugation (180,000xg, 45 min), was dissolved in 50 mM Tris/HCl, 10 mM MgCl₂, 10% [v/v] glycerol, pH 7.5) containing 1% [w/v] n-dodecyl- β -maltoside (DDM; Thermo Scientific, UAS). The *AwF*-ATP synthase was further purified using Ni²⁺-NTA-chromatography and Superdex HR 200 (GE Healthcare) column. The purity of the enzyme complex was assessed by SDS-PAGE and MALDI-MS [5].

2.2. Specimen preparation and EM data collection

The *AwF*-ATP synthase was diluted in 50 mM Tris/HCl, 10 mM MgCl₂, 10% [v/v] glycerol, 2 mM Pefabloc^{SC}, 0.05% [w/v] DDM, 0.8 mM dithiothreitol (DTT), pH 7.5 to 80 μ g/ml and a volume of 4 μ l of protein sample was applied to a glow discharged carbon coated copper transmission electron microscopy grid and stained with 2% (w/v) uranyl acetate. Electron micrographs were recorded on a Tecnai spirit T12 transmission electron microscope

(FEI) equipped with a 4K CCD camera (FEI) operated at a voltage of 120 kV at a calibrated magnification of 66,350x under low dose mode, with a dosage of 20-25 e⁻/Å². 90 micrographs were recorded at 0° angle for the enzyme complex.

2.3. Image processing, 3D map reconstruction and homologous model docking

Particle images were selected using EMAN2 [10]. The parameters for the microscope contrast transfer function were estimated by CTFFIND3 [11]. The 2D reference-free alignment was performed using the RELION v1.4. [12]. A total of 14,000 particles for AwF-ATP synthase were selected for 2D reference free alignment. The selection criteria were clear visibility of single particle and separation from neighboring particles. A 50 Å low-pass filtered *Escherichia coli* F-ATP synthase (PDB ID 5T4Q) [13] initial model was generated for 3D classification and class refinement using RELION v1.4 [12] to obtain the final reconstruction map. A homology model of the AwF₁F₀ ATP synthase was built by combining the structural information of the *c*-ring (PDB ID 4BEM) [9] and 3D models obtained by using the 3D structure prediction server, SWISS-MODEL [9, 14]. Model docking and visualization of the EM maps were performed in Chimera [5].

2.4. Molecular simulations

A structural model of AwF-ATP synthase was prepared as described in Bogdanović et al. [5] The assembly consists of the F₁ subunits α_3 , β_3 , γ , δ and ϵ as well the F₀ subunits *a*, *b*₂, *c*' and *c*₉. The initial structure was embedded into a bacterial membrane model patch consisting of POPE, POPI and PVCL2 lipids spanning 19x19 nm² using CHARMM-GUI [16]. The system was solvated in TIP3P water and neutralized using sodium and chloride counter-ions. The total salt concentration was set to 0.15 M, yielding a total system size of approximately 850,000 atoms. Interactions were described using the CHARMM36m [17] parameters.

Electrostatic interactions were described using the particle mesh Ewald (PME) method [18]. The Lennard-Jones interaction and PMEd direct-space cutoffs were set to 1.2 nm. The system was subjected to energy minimization, followed by a stepwise equilibration procedure, gradually reducing restraints on the protein using the templates provided by CHARMM-GUI. Equilibration and production runs were conducted using semi-isotropic pressure scaling with the Parrinello-Rahman barostat [19] at 1 bar and a velocity-rescaling thermostat at 300K [20]. The simulation time step was set to 2 fs after constraining bonds to hydrogen using the LINCS algorithm [21]. Frames were saved for analysis every 50 ps. 250 ns of production dynamics were obtained.

3. Results and discussion

3.1. Visualization of an intact AwF₁F₀ ATP synthase

The heterologously produced and purified AwF-ATP synthase was subjected to EM specimen preparation at a diluted concentration of 80 µg/ml and negatively-stained with 2% uranyl acetate. The negatively-stained enzyme yielded dispersed single particles, showing a preferred orientation on the electron microscope grids with their long axis lying in the plane of the carbon support film (Fig. 1A). A total of 14,000 images were subjected to the 2D reference-free alignment to extract the represented orientations. After two rounds of particle elimination and classification, 8,000 particles were used for 2D class averaging. The resulting class averages were well-defined and detailed (Fig. 1B). Principally, the 2D class averages demonstrate the side views of the *A. woodii* F-ATP synthase particles, reflecting the tripartite structure with an F₁ headpiece, a membrane-embedded domain, F₀, and the connecting central- and peripheral stalks (Fig. 2A). In addition, the few 2D class averages indicate top views, illustrating the hexameric arrangement of the F₁ headpiece, and bottom views, displaying the ring like feature of the F₀ domain (Fig. 2A). Besides that, the mass

contributing to the central stalk ($\gamma\epsilon$) and the peripheral stalk ($b_2\delta$), whose densities are indicated with arrows (Fig. 2A), were visible as well. The entire AwF-ATP synthase extends to a length of around 240 Å. The data confirm that a stable and well assembled AwF-ATP synthase was isolated.

3.2. Flexibility in the negative stain data of F-ATP synthase

The reference free 2D classes of AwF-ATP synthase in Figure 1B also revealed a variety of views, demonstrated by the different relative orientations of the F_1 and F_O parts. A range of 2D class averages (2, 3, 4, 7, 8, 9, 12 and 17 (Fig. 1B)) were observed with significant bending along the long axis of the complex. In comparison, the class averages 1, 6, 11, 14, 16 and 21 (Fig. 1B) revealed no bending along the long axis, while the classes 2, 3, 7, 8, 9, 12 and 17 reflected a different level of bending of the F_1 head piece by 1.5°, 14°, 23°, 21°, 21°, 18° and 28°, respectively. Furthermore, classes 2, 3, 4, 7, 8, 9, 12 and 17 highlight variations of bending of the F_O region by 17°, 11°, 18°, 21°, 14°, 21°, 31°, and 24°, respectively. Interestingly, the classes 2, 4, and 12 yielded a higher flexibility of the F_O - compared to the F_1 domain, while class 8 represented a higher flexibility of F_1 relative to F_O domain. It is interesting to note that in all flexible classes, the F_1 - and the F_O domain are oriented relative to each other as obvious in the 2D classes 9 and 12. Such data clearly demonstrate a significant flexibility in the intact AwF-ATP synthase, which nicely align with studies of other F-type ATP synthases and the corresponding V_1/V_O and A_1/A_O domains of the related vacuolar- and archaeal-type V-ATPases and A_1A_O ATP synthases, respectively, that showed significant flexibility between the catalytic- and membrane embedded parts [22-27]. This flexibility is thought to be important for the smooth transmission of power between the H^+/Na^+ driven $F_O/A_O/V_O$ motors and chemically active $F_1/A_1/V_1$ motor to function with high efficiency in a dynamic aqueous solution [22-24]. In addition, the flexibility is essential for the special and

structural reorganization of the enzyme within the inner membrane in general as well as dependent on metabolic conditions as most recently been visualized for the mitochondrial F-ATP synthase [28].

3.3. Overall structure

The final three-dimensional reconstruction (Fig. 2B) was calculated from a total of 8,000 negatively stained *Aw*F-ATP synthase particles (Fig. 1B). The resolution was determined to be 27 Å by the Fourier shell correlation criterion with a threshold of 0.143 times the noise correlation value (Supplementary Fig. 1). The 3D reconstruction of the intact *Aw*F-ATP synthase presented in a surface view clearly depicted the near spherical headpiece and a smaller basepiece consisting of the F_O domain. The head and base part were connected by two elements, a centrally localized axel and a relatively thin peripheral stalk. The dimensions of the F_1 headpiece, the central stalk domain, and the membrane part, F_O , are approximately 120 x 134, 46 x 80, and 78 x 112 Å, respectively (Fig. 2B). The final reconstruction enabled visualization of the peripheral stalk attached to the membrane-embedded F_O domain and, moving up to the distal end, of the water soluble F_1 domain on top of the $\alpha_3\beta_3$ crown forming the “tip-volume” (Fig. 2B). The gross structural features, such as shape and the coarse dimensions are similar to the intact *E. coli* and *Bacillus* PS3 F-ATP synthase structures [13, 27].

The resulting EM three-dimensional model was compared to the *E. coli* F-ATP synthase cryo-EM atomic model (PDB ID 5T4Q) [13]. The comparison illustrates that there is an overall good match of the molecule. The *Aw*F-ATP synthase map and the cryo-EM derived map of *E. coli* F-ATP synthase fit well, with a high correlation coefficient 0.91 at 30 Å. The difference map calculated between the *A. woodii* and *E. coli* F-ATP synthase did not show any major differences, except for the additional densities in the central stalk and F_O domain of

AwF-ATP synthase (Fig. 3). A cross-section at the central stalk reflects nicely that the additional mass in the 3D reconstruction (Supplementary Fig. 2) correlates to the rotary γ subunit.

3.4. EM structure of the unique hybrid engine

The AwF-ATP synthase is the only F_O - V_O hybrid engine capable of ATP synthesis, driven by a Na^+ electrochemical potential, described so far [3]. A homology model of the *A. woodii* F_1F_O ATP synthase was built by combining the structural information of the c -ring (PDB ID 4BEM) [9] and 3D models obtained as described earlier [5]. The generated structural model of the AwF-ATP synthase (Fig. 4A) docked well with the $\alpha_3\beta_3$ -headpiece, γ - ϵ central- and δ - b_2 peripheral stalk as well as the Na^+ translocating hybrid a -($c_{2/3}$) $_9$: c_1 F_O domain within the EM map in figure 4A. The rotor c -ring is composed of F_O -like $c_{2/3}$ subunits, with two transmembrane helices, and V_O -like c_1 subunit, with four transmembrane helices but only one Na^+ -binding site, in a stoichiometry of 9:1 (Fig. 4B). The central stalk subunits γ and ϵ fit well into the EM map with the unique $\gamma_{166-179}$ -loop, which occupies the additional mass observed for the central stalk of *A. woodii* F-ATP synthase (Fig. 3). The existence of an extra 14 and 34 amino acids noted within the γ subunit of mycobacteria ($\gamma_{166-179}$) [29, 30] and spinach chloroplast ($\gamma_{240-273}$) F-ATP synthases [25], respectively, has been described to be involved in a species-specific regulatory mechanisms [5]. The inserted loop region, predicted to form a random coil, could not be resolved in the recently determined structure of the soluble F_1 region (PDB ID: 6FOC) of *M. smegmatis* F-ATP synthase [31]. However, the loop deletion indicated that the unique γ -loop has a regulatory role inside the mycobacterial F-ATP synthase. In contrast, the chloroplast insertion comprises two β -hairpins arranged in an L-shape between the catalytic β subunit, which works as an auto-inhibitor of rotation in response to the chloroplast redox potential via a clash with the so-called DELSEED region of

subunit β [25]. Comparison of the *A. woodii* and chloroplast γ (PDB ID: 6FKH) subunits reveals that both the unique loops occupy a similar position in the EM map (Fig. 4B). In the predicted structural model of γ subunit, the $\gamma_{195-211}$ -loop adopts a random coil structure (Fig. 4B). However, on the basis of the structure prediction program PSIPRED [31], it is possible that this γ loop forms a combination of random coil (aa195-202) and α -helical (aa203-210) structure in *A. woodii* (Supplementary Fig. 3). Because of the close proximity of the *c*-ring loops, it can be predicted that the unique $\gamma_{195-211}$ -loop adopts conformational changes during $\gamma\epsilon$ -rotation, driven by the *c*-ring turbine, and provides sufficient coupling to synthesize ATP within the $\alpha_3\beta_3$ -headpiece. Such connectivity is indicated by the essentiality of the $\gamma_{195-211}$ -loop for Na^+ -translocation and consequently ATP synthesis within the *AwF*-ATP synthase [10].

3.5. Molecular simulations of the *AwF*-ATP synthase

Molecular simulations of *AwF*-ATP synthase (Fig. 4A) yielded results consistent with the dynamics observed in the electron micrographs. The bending dynamics observed by EM were apparent in our simulations, and the pivot point at the interface of the *c*-ring with the central stalk subunits γ and ϵ is consistent with experimental findings (Fig. 4C). A bending magnitude of up to 30 degrees is also consistent with the range of conformations observed experimentally (Fig. 4C).

At a more fine-grained level, the $\gamma_{195-211}$ -loop insertion characteristic of the *AwF*-ATP synthase subunit γ appears to be highly dynamic (Fig. 4B, Supplementary Movie 1). This dynamic loop could interact intermittently with the neighboring *c*-ring and the peripheral stalk subunit b_2 , but did not bind firmly to either. Moreover, the flexibility of this loop region was entirely confined to the loop itself, and did not affect the integrity of the remainder of the γ subunit (Fig. 4B).

Taken together, the EM studies described have resulted in the first 3D low resolution structure of an F₁V_O-hybrid F-ATP synthase with its characteristic $\alpha_3\beta_3$ -headpiece, a central and peripheral stalk both connecting the catalytic $\alpha_3\beta_3$ with the membrane-embedded F_O domain. The visualization of conformational flexibility of the two motor elements F₁ and F_O relative to each other were confirmed by molecular simulation studies, confirming that the enzyme complex is likely to be dynamic in its fluid physiological membrane environment. The essential extra $\gamma_{195-211}$ -loop of the A_wF-ATP synthase is highly dynamic and enables adaptation to the rotary loop elements of the *c*-ring turbine.

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Author contributions

K.N., R.G.H., P.J.B., V.M. and G.G. designed the experiments. K.N. and R.G.H. performed the experiments. K.N., R.G.H., and G.G. analyzed the data. Writing - Original Draft K.N., R.G.H. and G.G.; Writing - Review and Editing: all authors

Competing interests: Authors declare no competing interests.

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Figure Legends

Fig. 1. Negative stained EM of purified *AwF*-ATP synthase. (A) Electron micrograph showing the selected particles consisting of two visible domains. (B) The gallery reveals 2D class averages resulting from two rounds of reference free 2D classifications. Class averages demonstrate different bending levels of F_1 and F_0 relative to each other and highlight the flexibility of the *AwF*-ATP synthase.

Fig. 2. 3D reconstruction. (A) Classes showing the prominent structural features of the complex are magnified. In the side view of the particle where the F_1 and F_0 parts are clearly distinguished. *arrowheads* indicate the central stalk and the peripheral stalk connecting the F_1 (tip on the top of the F_1 domain) and F_0 part. The top view reveals the hexameric arrangement of the catalytic head piece and the ring like feature appeared from the bottom view. (B) Surface representation of the 3D reconstruction of the *M. smegmatis* F-ATP synthase refined to about 27 Å shown in side and top views. The arrows indicating the tip volume formed by the peripheral stalk. (C) Comparison and difference maps of the *A. woodii* and *E. coli* F-ATP synthase. Atomic model of *E. coli* F-ATP synthase (PDB ID: 5T4Q; [13]) docked into the EM map of *A. woodii* F-ATP synthase and subsequently used to calculate the difference map (*left panel*). An overlay of the *A. woodii* (grey surface) and *E. coli* (mesh) map and the green surface represents the difference map that is present in *A. woodii* map but not the *E. coli* map (*right panel*).

Fig. 3. The fitted model of the *AwF*-ATP synthase structure to the EM map. (A). The generated structural model of the *A. woodii* F-ATP synthase (Fig. 4A) docked well with its $\alpha_3\beta_3$ -headpiece, γ - ϵ central- and δ - b_2 peripheral stalk as well as the Na^+ translocating hybrid a -($c_{2/3}$) $_9$: c_1 F_0 domain (*right*). (B) Comparison of *A. woodii* (yellow) and chloroplast (grey)

subunit γ reveals that the unique loop occupies a similar position in the complex structure. The *A. woodii* and chloroplast γ loop are shown in *red* and *green* colors, respectively.

Fig. 4. Dynamics of the *Aw*F-ATP synthase. (A) The F-ATP synthase (molecular surface representation) is shown inserted within a lipid bilayer (*grey wireframe* format). The pivot point for bending motions observed in molecular simulations is located at the interface of the γ and ϵ subunits with the *c*-ring. This is consistent with the EM data. (B) Per-residue root mean square fluctuations (RMSF) of the *A. woodii* γ subunit backbone. The loop insertion characteristic of *A. woodii* is highly flexible, but does not affect the dynamics of the remainder of subunit γ . (C) Bending angle around the pivot measured throughout the simulation time.

(A)

(B)

Figures 1A-B

Figures 2A-C

Figures 3A-B

Figures 4A-C