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Cardiolipin interaction with subunit c of ATP synthase: Solid-state NMR characterization ☆

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ABSTRACT

The interaction of lipids with subunit c from F_1F_0 ATP synthase is studied by biophysical methods. Subunit c from both *Escherichia coli* and *Streptococcus pneumoniae* interacts and copurifies with cardiolipin. Solid state NMR data on oligomeric rings of F_0 show that the cardiolipin interacts with the c subunit in membrane bilayers. These studies offer strong support for the hypothesis that F_0 has specific interactions with cardiolipin. This article is part of a Special Issue entitled: NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

1. Introduction

Biological membranes, which play many key roles in cellular physiology, are often depicted as inert matrices anchoring essential proteins, for example as described in the fluid mosaic model [1]. However, there is a growing variety of evidence that the role of lipids goes beyond the one of an inert support. Arguably, tight interactions between membrane-embedded proteins and the phospholipids are implicitly required to maintain chemical potential gradients and keep the bilayer effectively sealed. Lipids have also been observed as structural members in membrane complexes including ion channels or membrane receptors [2–5]. The membrane-embedded F_1F_0-ATP synthase, which produces the majority of the ATP used by the cell, has been suggested to also bind lipids through its membrane-embedded portion F_0 [6–9]. The question of a preferential interaction with one particular type of lipid remains however up for discussions.

Several aspects of lipid–protein interactions have been probed in the past. The localization of the protein in the bilayer [10], the electrostatic component of the interaction [11], and the role of lipids in protein oligomerization [12] have been addressed by fluorescence correlation spectroscopy. Insights about the immersion of a protein in the bilayer can be probed by the combine use of protein labels and EPR spectroscopy [13]. Oriented solid-state NMR, with in particular the PISEMA experiment [14], is another powerful tool to characterize the immersion and tilt angle of a membrane protein inside the bilayer [15–18]. Magic-angle spinning (MAS) NMR offers the possibility to design an unlimited number of experiments [19,20]. The location of membrane-embedded systems can be assessed by measuring the exposure of the protein to paramagnetic labels [21,22]. Other experiments monitoring the efficiency of ^{1}H spin-diffusion between the lipids and the proteins, or water and the protein, have been successfully used to probe respectively the membrane-embedded or solvent-exposed portions of the protein [23–25]. The NMR-sensitive ^{31}P and ^{2}H nuclei are very accessible probes of the physical state of the membrane, allowing access to the effect of the protein on the phase and the dynamics of the lipids, and reciprocally [26–32]. These methods have been used to study several systems of highly relevant biological interest. One can quote for example the mechanism by which a virus merges with cell membranes [33], the action of antimicrobial peptides [34], and the implication of lipids in the mechanism of membrane proteins, such as the regulator cardiac muscle Phospholamban [35,36], or the potassium channel KcsA [37,38]. Implication of these interactions in non-viral pathologies, such as Huntingtin disease [39] or blood clotting [40], has also been investigated. But the question of specificity, implying a reconnaissance between one specific type of lipids and one specific site of the protein, remains difficult to evidence.

Depending on the strength of their interaction with the protein and their consequent rate of exchange, lipids have been referred to in different terms. The bulk lipids exchange very rapidly, and interact non-specifically with the protein mostly through the physical properties of the membrane such as its fluidity, lateral pressure, or the charge of its surface. The shell of lipids at the surface of the protein is usually

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referred to as annular lipids. The exchange rate of these lipids with the bulk is generally around 1 to 2.10^7 s^{-1} at 37 °C, which is at least a factor of 4 slower than the rate of exchange between two lipids of the bulk (8.10^7 s^{-1}) [41]. These exchange rates are all fast on an NMR timescale ($>10^4$ s^{-1}). Other lipids can be found buried within a membrane protein, in deep grooves in the protein surface, between transmembrane α-helices, or at the protein–protein interface of an oligomer. These lipids are referred to as non-annular lipids by contrast to the one at the annular sites [42,43]. They establish much stronger and specific interactions with the protein, as shown by their significantly lower exchange rate. Their longer lifetime at the binding site can allow their identification by X-ray crystallography [44]. Understanding lipid–protein interactions can have biological or medical implications. For example, non-annular lipids at the interface between transmembrane α-helices in helix bundles have been proposed to play a key role allowing relative motions of helices during protein function. Such motions can be modulated using hydrophobic inhibitors of Ca^{2+}-ATPase, small molecules that bind to the lipid binding cavities such as t-butylhydroquinone, cyclopiazonic acid, and thapsigargin [45,46].

ATP synthase is a molecular complex containing several subunits divided between the soluble F_1 portion and the membrane-embedded part F_0. The soluble F_1 part, where the enzymatic reaction occurs, has been well characterized. Far less is known about the transmembrane proton-pump F_0. Solid-state NMR turns out to be a very informative technique for the study of this membrane protein, as shown by recent studies of the active site structure [47]. Subunit c is a major constituent of F_0 and takes the form of an oligomeric ring. In the case of *Escherichia coli*, the c ring is made of 10 protomers of 79 amino acids each. The phospholipid composition of *E. coli* is dominated by phosphatidylethanolamine (69%), phosphatidylglycerol (19%), cardiolipin (CL) (6.5%) and other minor species such as phosphatidylserine and phosphatidic acid [48]. *Streptococcus pneumoniae* phospholipid composition was found to be dominated by phosphatidylglycerol and cardiolipin in a ratio close to 1:1 [49]. CL has a non-canonical structure: it consists of two phosphatidylglycerols connected by a glycerol moiety. It was shown to interact with or be essential for the functions of several mitochondrial proteins, including complexes involved in oxidative phosphorylation [50]. Complex I (NADH:ubiquinone oxidoreductase) and Complex III (ubihydroquinone:cytochrome c oxidoreductase) are inactive in the absence of CL [51,52]. The activity of ATP synthase (complex V) also depends on the presence of CL [9]. The existence of an interaction between CL and bovine ATP synthase, as well as *Thermus thermophilus* ATP synthase, was identified by solution NMR and mass spectrometry [9,8]. Here we study the c ring of *E. coli* and *S. pneumoniae* ATP synthases and their interactions with cardiolipin by MAS-NMR.

2. Materials and methods

2.1. Protein expression

A similar protocol was used for the preparation of *E. coli* and *S. pneumoniae* subunit c. The subunit c gene (atpE) cloned into a pET-17b vector was overexpressed in *E. coli* BL21(DE3) cells. After transformation, the cells were inoculated into 5 mL LB medium fractions containing 100 mg/L of ampicillin for over-night pre-culture. The cells were then grown in 1 L LB medium fractions containing 100 mg/L of ampicillin, at 37 °C with shaking at 250 rpm, until the OD_{600} (optical density at 600 nm) reached 0.8–1. The cells were pelleted and washed in P minimal medium [53], and then transferred into 250 mL fractions of P minimal medium containing 100 mg/L of ampicillin and enriched with 3 g/L $^{15}NH_4Cl$ and 4 g/L U-[^{13}C]glucose. After 30 min of growth at 37 °C with shaking at 250 rpm, the expression of subunit c was induced with 1 mM IPTG and continued for 18 h. The cells were then harvested and suspended in a 50 mM NH_4Ac buffer at pH 7 before extraction and purification.

2.2. Protein extraction and purification

The protein was extracted and purified thanks to organic solvent [54]. Six volumes (relative to the cell suspension) of 1:1 $CHCl_3$:MeOH were added, the cells were lysed by grinding with a blender for a few minutes, and then incubated at 4 °C for 2 h. The extract was then centrifuged and filtered to remove cell debris. Water and chloroform were then added to adjust the final $CHCl_3$:MeOH:H_2O ratio to 8:4:3. The aqueous (H_2O/MeOH) and organic ($CHCl_3$) phases were separated by centrifugation, the upper aqueous phase was removed by aspiration and the surface of the lower organic phase was washed several times with 3:47:48 $CHCl_3$:MeOH:H_2O fractions. The organic phase was then collected, one volume of HCl_3 was added and MeOH was adjusted to keep the protein from precipitating. This solution was then rotaevaporated to near dryness and suspended in a 5 mL fraction of 2:1 $CHCl_3$:MeOH. Subunit c was then precipitated by addition of 8 volumes of chilled ether, and left to precipitate at −20 °C for 48 h. The ether suspension was centrifuged at −4 °C and the resulting pellets were dried under nitrogen stream before being dissolved in a minimal amount of 2:1 $CHCl_3$:MeOH. The solution was then loaded on a CM52 cation-exchange column in the case of *E. coli* subunit c and a DE52 column in the case of *S. pneumoniae* subunit c. The column was washed with loading buffer and 1:1 $CHCl_3$:MeOH and subunit c was then eluted with 5:5:1 $CHCl_3$:MeOH:H_2O fractions. The yield of protein was about 50 mg per liter minimum media.

2.3. Reconstitution in lipid bilayers

Only *E. coli* subunit c was reconstituted in lipid bilayers. The subunit c ring was reconstituted in POPC liposomes with a protein-to-lipid ratio of 1:1 (w:w). 10 mg of protein and 100 mg of β-octylpyranoside detergent were mixed with 10 mg of POPC in $CHCl_3$. The solvent was evaporated under nitrogen to form a film of mixed protein-detergent-lipids. The dried film was then dissolved in aqueous buffer (20 mM Tris–HCl, 10 μM DTT, 20% glycerol, 0.03% NaN_3, pH 8) thanks to 10 cycles of 20 min of freeze–thaw cycles at 40 °C with sonication and −20 °C. The solution was then diluted to reach the CMC of the detergent and dialyzed against 8 L of buffer (10 mM HEPES, 10% glycerol, 0.1 mM TCEP, 0.2% NaN_3, pH 5) at room temperature for 48 h. The precipitate was then collected by centrifugation and packed into Bruker 3.2 mm rotors.

2.4. Solid-state NMR spectroscopy

The solid-state experiments were carried out on a Bruker Avance 900 MHz spectrometer using a 3.2 mm triple tuned (^{1}H, ^{13}C, ^{15}N) E-Free CP-MAS probe with a 20 kHz MAS frequency. The ordered temperature was set to 260 K. The DARR experiment was acquired with 100 ms mixing times [55]. Magnetization transfer between ^{1}H and ^{13}C nuclei was performed with an echo of 60 ms during the INEPT experiment [56]. The signal was acquired on the ^{13}C channel under Spinal-64 heteronuclear decoupling [57] with a r.f. field strength of 90 kHz. Quadrature detection was obtained with TPPI. The maximum acquisition time was set to 7 ms in t_1 and 9 ms in t_2 for the DARR experiment. The maximum acquisition time was set to 12 ms in t_1 and 9 ms in t_2 for the INEPT experiment. Data were processed using zero-filling up to 2048 points in t_1, and 4096 points in t_2, with a square cosine filter with a baseline correction in both dimensions. Processing was performed using the software Topspin 3.1. Referencing was made with respect to DSS, based on an external reference of adamantane.

2.5. Isolation of lipids

Lipids were isolated from the purified protein samples by lyophilization followed by dissolution in methanol. Contrary to phospholipids, subunit c is indeed not soluble in methanol. The isolated lipids were then analyzed by solution NMR and mass spectrometry.