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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.

2.6. Solution NMR spectroscopy

The solution experiments were carried out on a Varian Inova 600 MHz spectrometer using a CryoHCN probe. All HSQC were acquired with a maximum acquisition time of 12 ms in t_1 and 140 ms in t_2. Data were processed 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, assuming a difference between TMS and DMS of 1.9 ppm.

2.7. Mass spectrometry

The lipids isolated from *S. pneumoniae* subunit c sample were analyzed by ESI–MS on a Thermo Finnigan LTQ mass spectrometer using flow injection analysis and negative ion mode. The lipid extract (1.4 to 14 μM) in chloroform was re-suspended in 50% methanol at a ratio of 1:5 and 10 μL was injected into a continuous flow of the same solvent (50% methanol) at 20 μL/min. The ESI source spray voltage was at 5 kV and the heated capillary at 275 °C. ESI–MS data were collected at profile mode, 2 microscans, 100 ms maximum injection time and from 200 or 600–1800 m/z.

3. Results

3.1. Evidence of an interaction between the subunit c of ATP synthase and lipids

The spectra of the experiments recorded at a proton field of 900 MHz on the uniformly enriched *E. coli* subunit c show some correlation peaks which don't belong to the protein and that we assign to lipids. In the high resolution magic angle spinning ^{13}C–^{13}C spectra of uniformly enriched c subunit reconsitituted in lipid bilayers, some correlation peaks can be unambiguously assigned to cardiolipin. This observation was made primarily based on correlations in a ^{13}C–^{13}C correlation DARR [55] spectrum (Fig. 1) recorded at an effective temperature of 280 K, a temperature for which the POPC bilayer is expected to be in a liquid disordered Lα phase. In this spectrum, the region between 64 and 74 ppm clearly shows several correlation peaks: an isolated cross peak between 69.3 ppm and 72.5 ppm, and three partially resolved cross peaks between 65.9 ppm and 73.4 ppm (Table 1). The chemical shifts of these peaks were respectively assigned to the central and peripheral glycerol groups of CL (Table 1) [58]. Based on the strength of the cross peaks, it is very likely that this signal arises from a molecule of uniformly and highly ^{13}C-isotopically enriched CL from the *E. coli* growth. No other strong cross-peaks were observed for these samples in the chemical shift range corresponding to the glycerol functionalities of phospholipids, suggesting that CL is the only endogenous lipid present in large (stoichiometric or greater) amounts.

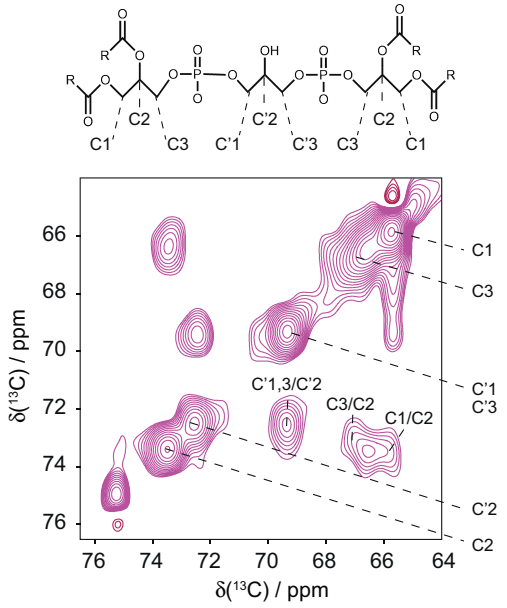

Fig. 1. 64–76 ppm region of the ^{13}C–^{13}C DARR correlation spectrum of a sample of *E. coli* ATP synthase subunit c reconstituted in POPC bilayers. The annotated correlation peaks are assigned to endogenous CL, whose general structure is shown on top of the spectrum. The spectrum was recorded on a 900 MHz spectrometer, at a spinning frequency of 20 kHz and a temperature of 280 K, with a mixing time of 150 ms.

3.2. Identification of a specific interaction with cardiolipin

The specificity of the interaction between subunit c and cardiolipin was verified on two levels: the presence of cardiolipin was confirmed and the absence of other lipids was proved.

The assignment of CL was confirmed by ^{1}H–^{13}C correlation NMR spectroscopy both in the solid-state and in solution. In the solid-state, we recorded an INEPT spectrum on the sample of *E. coli* subunit c reconstituted in POPC (Fig. 2). In contrast to the DARR spectra described above, INEPT spectra are based on long through-bond ^{1}H–^{13}C magnetization transfers and show enhanced signals for most mobile nuclei with longer transverse coherence life times. The INEPT spectrum of the c subunit preparations at 280 K displays ^{1}H and ^{13}C chemical shift correlations assignable to the lipids, whereas the signals from the less mobile protein are not visible. The strongest signals are from natural abundance ^{13}C nuclei in the POPC molecules from the reconstituted membrane. Weaker peaks, whose ^{13}C chemical shifts correspond to the ones assigned to CL on the DARR spectrum, are also clearly visible. The ^{1}H chemical shifts and the additional ^{13}C shifts are in good agreement with those reported for CL previously (Table 1). The presence of the CL in INEPT spectrum suggests that some of the CL molecules are in rapid conformational exchange or rotate rapidly on the microsecond timescale. We assign these peaks to molecules of CL which exchanged with the bulk, or to some residual mobility in the bound lipids.

The assignment of CL and the absence of other lipids were further confirmed by solution NMR on isolated lipids samples (Fig. 3). ^{13}C–^{1}H HSQC experiments were recorded on commercial samples of POPC (Fig. 3a), POPG (Fig. 3b), CL (Fig. 3c) and compared to samples of lipids isolated from *S. pneumoniae* subunit c (Fig. 3d) and *E. coli* subunit c (Fig. 3e). Even if these spectra were proton-detected, they are displayed in a way allowing a direct comparison with the solid-state INEPT spectrum of Fig. 2. A close-up view of the glycerol region (Fig. 3f) shows a perfect superposition of the *E. coli* and *S. pneumoniae* lipids spectra with the commercial CL spectrum. This observation further confirms the assignment of the endogenous lipids to CL. The glycerol region displayed in Fig. 3f also shows that POPC and POPG are absent from the natural lipids spectra. In the natural lipids spectra, the absence of peaks at 74–4 ppm, 65–3.9 ppm and 69–3.9 ppm, 62–4.2 ppm, 56–3.4 ppm shows the absence of POPG and POPC respectively. This confirms the specificity of the subunit c interaction with cardiolipin.

The nature of the endogeneous lipids was further investigated by mass spectrometry. The ESI–MS spectra of a sample of lipids isolated from *S. pneumoniae* subunit c are shown in Fig. 4. The general pattern

Table 1
^{1}H and ^{13}C chemical shifts of $E^{1}H$ and ^{13}C chemical shifts of $E^{1}H$ and ^{13}C chemical shifts of $E^{1}H$ and ^{13}C chemical shifts of *E. Coli* CL[a].

Component moiety	C numbering	System	^{1}H δ (ppm)	^{13}C δ (ppm)	Agreement with the literature (ppm)
Outer glycerol	C-1	CH_2O	3.8	65.9	−0.3/-0.1
	C-2	CHO	5.6	73.4	+0.4/-0.8
	C-3	CH_2OP	3.9	67.0	−0.1/−0.1
Central glycerol	C′-1 + C′-3	CH_2OP	4.1	69.3	+0.2/−0.1
	C′-2	CHO	4.2	72.5	+0.5/−0.6

[a] All chemical shifts are referenced with respect to DSS.

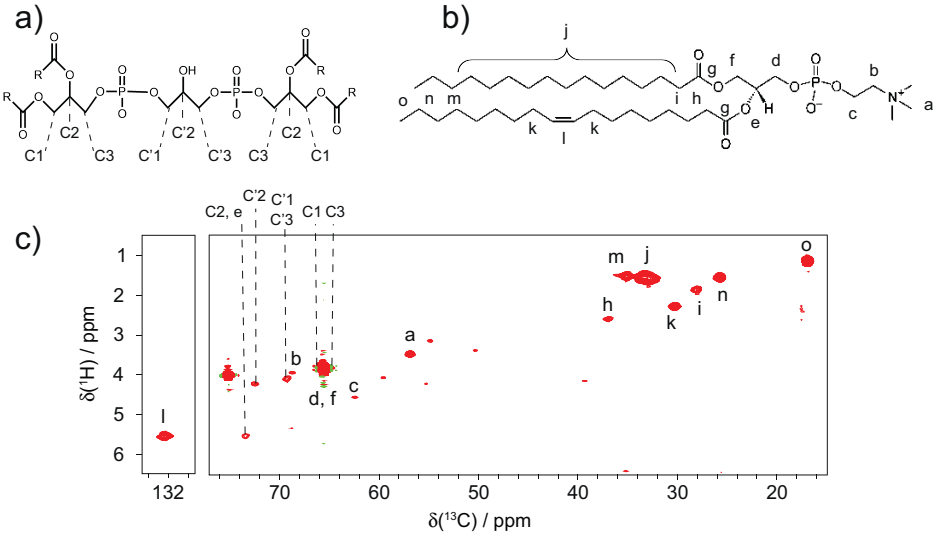

Fig. 2. ^{1}H–^{13}C INEPT spectrum of a sample of *E. coli* ATP synthase subunit c reconstituted in POPC bilayers. Peaks are assigned to the natural abundance commercial POPC and endogenous CL. The spectrum was recorded on a 900 MHz spectrometer, at a spinning frequency of 20 kHz and a temperature of 280 K.

is very similar to the one found in the literature for cardiolipin [59]. The most intense peak at m/z 1428 can be assigned to the $[M-H]^-$ species. It brings further information regarding the dominant lipid hydrophobic chain since 1428 corresponds exactly to the mass of a (16:0/16:0)(16:0/16:0)-CL enriched in ^{13}C. The breadth of the peak is attributed to partial isotope labeling and distribution of the lipid chain length. The second

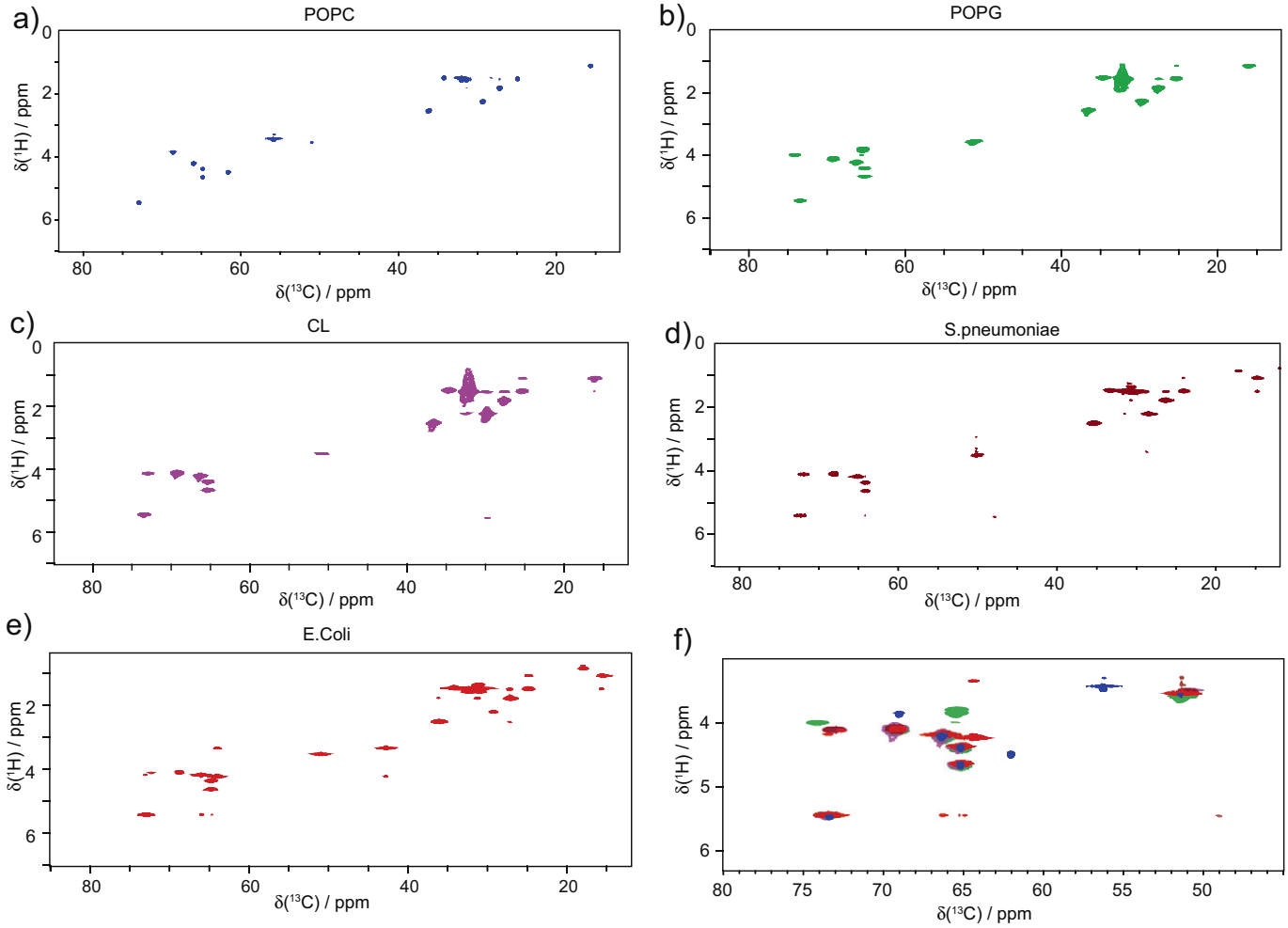

Fig. 3. ^{13}C–^{1}H HSQC spectra of (a) commercial POPC, (b) commercial POPG, (c) commercial CL and lipids isolated from (d) *S. pneumoniae* and (e) *E. coli* subunit c. (f) Close-up view of the glycerol region with a superposition of the previous spectra in the same color-code (blue: POPC, green: POPG, purple: CL, dark-red: *S. pneumoniae* lipids, red: *E. coli* lipids).

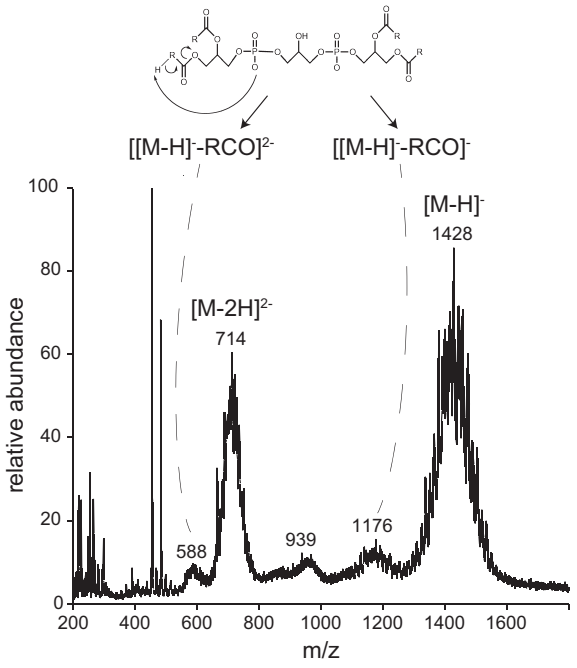

Fig. 4. ESI–MS spectra of lipids isolated from *S. pneumoniae* subunit c isotopically labeled in ^{13}C. The assignment of the dominant peaks, as well as the main fragmentation process is displayed on the figure.

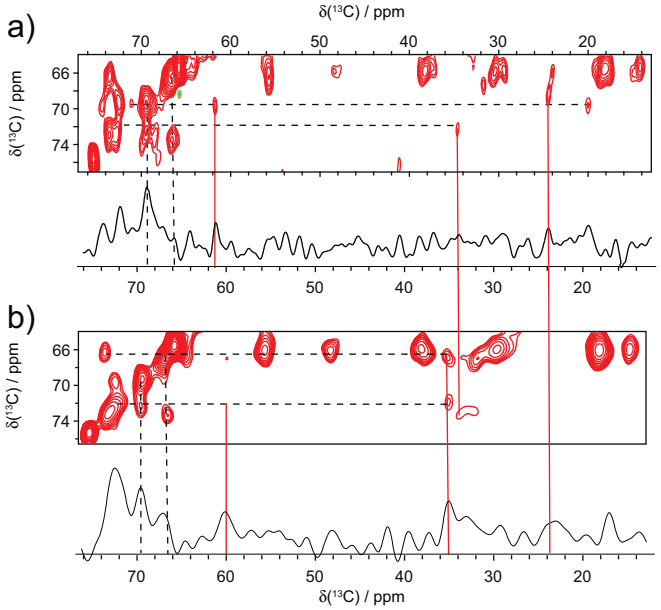

Fig. 5. ^{13}C–^{13}C DARR correlation spectrum of a sample of *E. coli* ATP synthase subunit c reconstituted in POPC bilayers, with a mixing time of 350 ms (a) and 450 ms (b). The 65.9 and 72.5 ppm peaks of endogenous CL correlate with peaks at 60.3, 35.0 and 23.9 ppm. The rows extracted at 72.5 ppm also show correlation peaks above the noise level at these three chemical shifts. The spectra were recorded on a 900 MHz spectrometer, at a spinning frequency of 20 kHz and a temperature of 280 K.

major peak at m/z 724 is assigned to the $[M-2H]^{2-}$ species. The loss of fatty acyl ketene as shown in Fig. 4 leads to singly and doubly-charged fragment ions at m/z 1176 (1428–253) and m/z 588 (1428–253)/2 respectively. This overall aspect of the MS spectrum confirms the strong and specific interaction of ATP synthase subunit c with cardiolipin and brings additional insight on the nature of the dominant hydrophobic chain.

3.3. Evidence of a non-annular interaction

^{3}C–^{13}C DARR spectra with very long mixing times can be used to detect long-distance correlations peaks (up to 6–7 Å [60]). DARR spectra of 350 and 450 ms mixing times recorded on the *E. coli* subunit c sample reveal interesting potential lipid–protein contacts (Fig. 5). Specifically peaks at 23.9 ppm, 35.0 ppm and 60.3 ppm correlate with the peak at 72.5 ppm uniquely identified as C′2 nuclei of the central glycerol of CL. These correlations may support a recent hypothesis of an interaction between CL and Lys34 [61,62], since the chemical shifts (60.3 ppm, 35.0 ppm and 23.9 ppm) are consistent with possible assignment of Lys Ca, Cd and Cg and Lys34 is the only Lys present in the *E. coli* subunit c sequence. This last result should be taken with high precaution though, since the 72.5–60.3 ppm correlation is very weak and the 35.0 ppm peak is ambiguously assigned due to similar chemical shifts of lipids hydrophobic chains.

Even if the exact localization of the interaction is uncertain, two criteria indicate that the CL is "non-annular" or bound to the protein. First, we clearly demonstrated that they are endogenous molecules of CL that co-purified with the c subunit protein. This in turn implies that some CL in the *E. coli* cells were bound tightly enough to subunit c to resist the chloroform extraction used during the preparation of the sample. During this protocol, the c protomer is first isolated and the ring is then reconstituted in detergents. These lipids are therefore probably not simply trapped inside the ring, but are likely involved in a strong interaction with the c protomer. It also implies that the exchange rate of these lipids is slow on an NMR time scale. We conclude that subunit c of ATP synthase contains some non-annular binding sites that are specific for CL. As previously mentioned, the existence of an interaction between the protein and this lipid has recently been suggested by Walker [61]. His hypothesis involves a methylated K34. Our data also are suggestive of the structural and functional involvement of this residue, perhaps facilitating the motion of the c-ring necessary to the proton pump.

We have shown using solid-state NMR that the subunit c of ATP synthase is involved in a non-annular interaction with CL. This interaction was proved to be specific and conserved among several organisms (*E. coli* and *S. pneumoniae*). These observations overall emphasize the importance of structural studies in a bilayer environment as close as possible to its native one, preserving the activity of ATP synthase [9]. It also opens the possibility to consider using hydrophobic inhibitors binding to annular lipids site of subunit c of ATP synthase for medical applications. Subunit c is notably known for being the drug target of major diseases such as tuberculosis.

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References

[1] S.J. Singer, G.L. Nicolson, The fluid mosaic model of the structure of cell membranes, Science 80 (175) (1972) 720–731.
[2] A.M. Powl, J.M. East, A.G. Lee, Anionic phospholipids affect the rate and extent of flux through the mechanosensitive channel of large conductance MscL, Biochemistry 47 (2008) 4317–4328.
[3] X. Tao, J.L. Avalos, J. Chen, R. Mackinnon, Crystal structure of the eukaryotic strong inward-rectifier K + channel Kir22 at 31 A resolution, Science 326 (2009) 1668–1674.
[4] P. Marius, M. Zagnoni, M.E. Sandison, J.M. East, H. Morgan, A.G. Lee, Binding of anionic lipids to at least three nonannular sites on the potassium channel KcsA is required for channel opening, Biophys. J. 94 (2008) 1689–1698.

[5] S.-Y. Lee, A. Lee, J. Chen, R. Mackinnon, Structure of the KvAP voltage-dependent K + channel and its dependence on the lipid membrane, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15441–15446.
[6] T. Murata, I. Yamato, Y. Kakinuma, A.G.W. Leslie, J.E. Walker, Structure of the rotor of the V-Type Na + −ATPase from *Enterococcus hirae*, Science 308 (2005) 654–659.
[7] B. Oberfeld, J. Brunner, P. Dimroth, Phospholipids occupy the internal lumen of the c ring of the ATP synthase of *Escherichia coli*, Biochemistry 45 (2006) 1841–1851.
[8] M. Zhou, N. Morgner, N.P. Barrera, M. Zhou, N. Morgner, N.P. Barrera, a. Politis, S.C. Isaacson, D. Matak-Vinkovic, T. Murata, R.a. Bernal, D. Stock, C.V. Robinson, Mass spectrometry of intact V-type ATPases reveals bound lipids and the effects of nucleotide binding, Science 80 (334) (2011) 380–385.
[9] K.S. Eble, W.B. Coleman, R.R. Hantgan, C.C. Cunningham, Tightly associated cardiolipin in the bovine heart mitochondrial ATP synthase as analyzed by 31P nuclear magnetic resonance spectroscopy, J. Biol. Chem. 265 (1990) 19434–19440.
[10] D. Marsh, Immobilized lipid in acetylcholine receptor-rich membranes from torpedo marmorata, Proc. Natl. Acad. Sci. 75 (1978) 4329–4333.
[11] Y. Hong, J. Muenzner, S.K. Grimm, E.V. Pletneva, Origin of the conformational heterogeneity of cardiolipin-bound cytochrome C, J. Am. Chem. Soc. 134 (2012) 18713–18723.
[12] V. Betaneli, E.P. Petrov, P. Schwille, The role of lipids in VDAC oligomerization, Biophys. J. 102 (2012) 523–531.
[13] Y. Shin, C. Levinthal, F. Levinthal, W. Hubbell, Colicin E1 binding to membranes: time-resolved studies of spin-labeled mutants, Science 80 (259) (1993) 960–963.
[14] A. Ramamoorthy, S.J. Opella, Two-dimensional chemical shift/heteronuclear dipolar coupling spectra obtained with polarization inversion spin exchange at the magic angle and magic-angle sample spinning (PISEMAMAS), Solid State Nucl. Magn. Reson. 4 (1995) 387–392.
[15] S. Lambotte, P. Jasperse, B. Bechinger, Orientational distribution of alpha-helices in the colicin B and E1 channel domains: a one and two dimensional 15 N solid-state NMR investigation in uniaxially aligned phospholipid bilayers, Biochemistry 37 (1998) 16–22.
[16] Y. Kim, K. Valentine, S.J. Opella, S.L. Schendel, W.A. Cramer, Solid-state NMR studies of the membrane-bound closed state of the colicin E1 channel domain in lipid bilayers, Protein Sci. 7 (1998) 342–348.
[17] J. Wang, J. Denny, C. Tian, S. Kim, Y. Mo, F. Kovacs, Z. Song, K. Nishimura, Z. Gan, R. Fu, J.R. Quine, T.A. Cross, Imaging membrane protein helical wheels, J. Magn. Reson. 144 (2000) 162–167.
[18] M. Ouellet, M. Auger, Structure and membrane interactions of antimicrobial peptides as viewed by solid-state NMR spectroscopy, Annu. REPORTS NMR Spectrosc. VOL 63 (63) (2008) 1–21.
[19] T.A. Cross, D.T. Murray, A. Watts, Helical membrane protein conformations and their environment, Eur. Biophys. J. 42 (2013) 731–755.
[20] P.T.F. Williamson, Solid-state NMR for the analysis of high-affinity ligand/receptor interactions, Concepts Magn. Reson. Part A 34A (2009) 144–172.
[21] G. Gröbner, C. Glaubitz, A. Watts, Probing membrane surfaces and the location of membrane-embedded peptides by (13)C MAS NMR using lanthanide ions, J. Magn. Reson. 141 (1999) 335–339.
[22] Y. Su, F. Hu, M. Hong, Paramagnetic Cu(II) for Probing Membrane Protein Structure and Function: Inhibition Mechanism of the Influenza M2 Proton Channel, 2012.
[23] D. Huster, X. Yao, M. Hong, Membrane protein topology probed by 1H spin diffusion from lipids using solid-state NMR, Spectroscopy (2002) 9967–9971.
[24] A. Kijac, A.Y. Shih, A.J. Nieuwkoop, K. Schulten, S.G. Sligar, C.M. Rienstra, Lipid–Protein Correlations in Nanoscale Phospholipid Bilayers Determined by Solid-State Nuclear Magnetic Resonance †, 2010. 9190–9198.
[25] D. Krepkiy, K. Gawrisch, K.J. Swartz, structural interactions between lipids, water and S1–S4 voltage-sensing domains, J. Mol. Biol. 423 (2012) 632–647.
[26] E. Oldfield, M. Meadows, M. Glaser, Deuterium magnetic resonance spectroscopy of isotopically labeled mammalian cells, J. Biol. Chem. 251 (1976) 6147–6149.
[27] S.J. Kohler, M.P. Klein, Phosphorus-31 nuclear magnetic resonance chemical shielding tensors of phosphorylethanolamine, lecithin, and related compounds: applications to head-group motion in model membranes, Biochemistry 15 (1976) 967–973.
[28] G.W. Stockton, K.G. Johnson, K.W. Butler, A.P. Tulloch, Y. Boulanger, I.C.P. Smith, J.H. Davis, M. Bloom, Deuterium NMR study of lipid organisation in *Acholeplasma laidlawii* membranes, Nature 269 (1977) 267–268.
[29] R.G. Griffin, L. Powers, P.S. Pershan, Head-group conformation in phospholipids: a 31 P nuclear magnetic resonance study of oriented monodomain dipalmitoylphosphatidylcholine bilayers, Biochemistry 17 (1978) 2718–2722.
[30] Biological membrane structure by solid-state NMR, Curr. Issues Mol. Biol. 2 (2000) 119–124.
[31] P.T. Williamson, J.A. Watts, G.H. Addona, K.W. Miller, A. Watts, Dynamics and orientation of N + (CD3)3-bromoacetylcholine bound to its binding site on the nicotinic acetylcholine receptor, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 2346–2351.
[32] M.T. Eddy, T. Ong, L. Clark, O. Teijido, P.C.A. Van Der Wel, R. Garces, G. Wagner, T.K. Rostovtseva, R.G. Griffin, Lipid Dynamics and Protein − Lipid Interactions in 2D Crystals Formed with the β-Barrel Integral Membrane Protein VDAC1, 2012.
[33] H. Yao, M. Hong, Membrane-dependent conformation, dynamics, and lipid interactions of the fusion peptide of the paramyxovirus PIV5 from solid-state NMR, J. Mol. Biol. 425 (2013) 563–576.
[34] Y. Su, S. Li, M. Hong, Cationic membrane peptides: atomic-level insight of structure-activity relationships from solid-state NMR, Amino Acids 44 (2013) 821–833.
[35] N.J. Traaseth, L. Shi, R. Verardi, D.G. Mullen, G. Barany, G. Veglia, Structure and topology of monomeric phospholamban in lipid membranes determined by a hybrid solution and solid-state NMR approach, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 10165–10170.
[36] R. Verardi, L. Shi, N.J. Traaseth, N. Walsh, G. Veglia, Structural topology of phospholamban pentamer in lipid bilayers by a hybrid solution and solid-state NMR method, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 9101–9106.
[37] P. Marius, M.R.R. De Planque, P.T.F. Williamson, Biochimica et Biophysica Acta probing the interaction of lipids with the non-annular binding sites of the potassium channel KcsA by magic-angle spinning NMR, Biochim. Biophys. Acta Biomembr. 1818 (2012) 90–96.
[38] M. Weingarth, A. Prokofyev, E. a W. van der Cruijsen, D. Nand, A.M.J.J. Bonvin, O. Pongs, M. Baldus, Structural determinants of specific lipid binding to potassium channels, J. Am. Chem. Soc. 135 (2013) 3983–3988.
[39] M. Michalek, E.S. Salnikov, S. Werten, B. Bechinger, Membrane interactions of the amphipathic amino terminus of huntingtin, Biochemistry 52 (2013) 847–858.
[40] J.H. Morrissey, E. Tajkhorshid, C.M. Rienstra, Nanoscale studies of protein–membrane interactions in blood clotting, J. Thromb. Haemost. 9 (Suppl. 1) (2011) 162–167.
[41] D. Marsh, Protein modulation of lipids, and vice-versa, in membranes, Biochim. Biophys. Acta 1778 (2008) 1545–1575.
[42] A.C. Simmonds, J.M. East, O.T. Jones, E.K. Rooney, J. Mcwhirter, A.G. Lee, Annular and non-annular binding sites on the (Ca2+ + Mg2+)-ATPase, Biochim. Biophys. Acta Biomembr. 693 (1982) 398–406.
[43] A. Lee, Lipid–protein interactions in biological membranes: a structural perspective, Biochim. Biophys. Acta Biomembr. 1612 (2003) 1–40.
[44] M.G. Bertero, R.A. Rothery, M. Palak, C. Hou, D. Lim, F. Blasco, J.H. Weiner, N.C.J. Strynadka, Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A, Nat. Struct. Biol. 10 (2003) 681–687.
[45] A.-M.L. Winther, H. Liu, Y. Sonntag, C. Olesen, M. Le Maire, H. Soehoel, C.-E. Olsen, S.B. Christensen, P. Nissen, J.V. Møller, Critical roles of hydrophobicity and orientation of side chains for inactivation of sarcoplasmic reticulum Ca2 + −ATPase with thapsigargin and thapsigargin analogs, J. Biol. Chem. 285 (2010) 28883–28892.
[46] M. Takahashi, Y. Kondou, C. Toyoshima, Interdomain communication in calcium pump as revealed in the crystal structures with transmembrane inhibitors, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 5800–5805.
[47] S.-J. Kang, Y. Todokoro, I. Yumen, B. Shen, I. Iwasaki, T. Suzuki, A. Miyagi, M. Yoshida, T. Fujiwara, H. Akutsu, Active-site structure of the thermophilic foc-subunit ring in membranes elucidated by solid-state NMR, Biophys. J. 106 (2014) 390–398.
[48] G.F. Ames, Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism, J. Bacteriol. 95 (1968) 833–843.
[49] M.-C. Trombe, M.-A. Lanéelle, G. Lanéelle, Lipid composition of aminopterin-resistant and sensitive strains of *Streptococcus pneumoniae* effect of aminopterin inhibition, Biochim. Biophys. Acta Lipids Lipid Metab. 574 (1979) 290–300.
[50] T.J. Pinheiro, a Watts, Resolution of individual lipids in mixed phospholipid membranes and specific lipid–cytochrome c interactions by magic-angle spinning solid-state phosphorus-31 NMR, Biochemistry 33 (1994) 2459–2467.
[51] M.S. Sharpley, R.J. Shannon, F. Draghi, J. Hirst, Interactions between phospholipids and NADH:ubiquinone oxidoreductase (complex I) from bovine mitochondria, Biochemistry 45 (2006) 241–248.
[52] M. Zhang, E. Mileykovskaya, W. Dowhan, Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria, J. Biol. Chem. 280 (2005) 29403–29408.
[53] F.W. Studier, Protein production by auto-induction in high-density shaking cultures, Protein Expr. Purif. 41 (2005) 207–234.
[54] F. Valiyaveetil, J. Hermolin, R.H. Fillingame, pH dependent inactivation of solubilized F1F0 ATP synthase by dicyclohexylcarbodiimide: pK(a) of detergent unmasked aspartyl-61 in *Escherichia coli* subunit c, Biochim. Biophys. Acta 1553 (2002) 296–301.
[55] K. Takegoshi, S. Nakamura, T. Terao, [sup 13]C–[sup 1]H dipolar-driven [sup 13]C–[sup 13]C recoupling without [sup 13]C rf irradiation in nuclear magnetic resonance of rotating solids, J. Chem. Phys. 118 (2003) 2325.
[56] C.A. Fyfe, K.C. Wong-Moon, Y. Huang, H. Grondey, INEPT experiments in solid-state NMR, J. Am. Chem. Soc. 117 (1995) 10397–10398.
[57] A. Bielecki, A.C. Kolbert, M.H. Levitt, Frequency-switched pulse sequences: homonuclear decoupling and dilute spin NMR in solids, Chem. Phys. Lett. 155 (1989) 341–346.
[58] E. Kraffe, P. Soudant, Y. Marty, N. Kervarec, P. Jehan, Evidence of a tetradocosahexaenoic cardiolipin in some marine bivalves, Lipids 37 (2002) 507–514.
[59] F.-F. Hsu, J. Turk, E.R. Rhoades, D.G. Russell, Y. Shi, E.A. Groisman, Structural characterization of cardiolipin by tandem quadrupole and multiple-stage quadrupole ion-trap mass spectrometry with electrospray ionization, J. Am. Soc. Mass Spectrom. 16 (2005) 491–504.
[60] J. Yang, S. Paramasivam, D. Marulanda, M. Cataldi, M.L. Tasayco, T. Polenova, Magic angle spinning NMR spectroscopy of thioredoxin reassemblies, Magn. Reson. Chem. 45 (Suppl. 1) (2007) S73–S83.
[61] L.A. Baker, I.N. Watt, M.J. Runswick, J.E. Walker, J.L. Rubinstein, Arrangement of subunits in intact mammalian mitochondrial ATP synthase determined by cryo-EM, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 11675–11680.
[62] R. Chen, Lysine 43 is trimethylated in subunit c from bovine mitochondrial ATP synthase and in storage bodies associated with batten disease, J. Biol. Chem. 279 (2004) 21883–21887.