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Phospholipid composition and organization of cytochrome *c* oxidase preparations as determined by ^{31}P-nuclear magnetic resonance

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The molecular organization as well as the composition of the phospholipids in cytochrome *c* oxidase preparations (bovine heart) were investigated by ^{31}P-nuclear magnetic resonance. In the so-called 'lipid-rich' preparation the lipids were found to form a fluid bilayer around the enzyme since the ^{31}P-NMR spectrum was characteristic of a fast, axially symmetric motion of the phosphate groups with a chemical shift anisotropy of $\Delta\sigma = -45$ ppm. In contrast, the 'lipid-depleted' cytochrome *c* oxidase gave rise to a broader spectrum where the motion of the phospholipids was no longer axially symmetric. Nevertheless, the total width of the spectrum was still considerably narrower than observed for immobilized phospholipids in solid crystals. Both enzyme preparations were dissolved in 1% detergent solution and used for high-resolution ^{31}P-NMR spectroscopy. Narrow lines of about 20 Hz linewidth were obtained for both types of enzyme preparations, and well-resolved resonances could be assigned to cardiolipin, phosphatidylethanolamin and phosphatidylcholine. The major differences between lipid-rich and lipid-depleted cytochrome *c* oxidase were the absolute amount of phospholipid associated with the protein and the relative contribution of the individual lipid classes to the ^{31}P-NMR spectrum. For lipid-rich cytochrome *c* oxidase about 130 molecules phospholipid were bound per enzyme (approx. 11 cardiolipins, 54 phosphatidylethanolamines and 64 phosphatidylcholines). For lipid-depleted cytochrome *c* oxidase only 6–18 lipids were bound per enzyme (1 or 2 cardiolipins, 3–8 phosphatidylethanolamines and 2–8 phosphatidylcholines). In contrast to earlier suggestions that cardiolipin is the only remaining lipid in lipid-depleted cytochrome *c* oxidase, the ^{31}P-NMR studies demonstrate that all three lipids remain associated with the protein.

Introduction

Cytochrome *c* oxidase is an intrinsic membrane protein which spans the membrane completely (for a review on structure and topography see Ref. 1). For maximum enzyme activity a fluid lipid environment appears to be necessary, but differing views exist on the exact nature of the lipid-protein interaction. Spin-label EPR experiments revealed the existence of two motionally distinct environments where phospholipids in direct contact with the protein appeared to be 'immobilized' compared to the rest of the lipids [2–6]. However, this picture was not confirmed by subsequent NMR studies with reconstituted cytochrome *c* oxidase membranes, which showed only a single time-averaged lipid environment [7–13]. This discrepancy may be explained by the different time scales of EPR and NMR [13]. A second point of interest is the lipid specificity of cytochrome *c* oxidase. The lipid composition of the inner mitochondrial membrane and of lipid-rich preparations of cytochrome *c* oxidase (bovine heart) is rather similar with about 38 wt.% phosphatidylcholine, 30 wt.% phosphatidylethanolamine and 18 wt.% cardiolipin as the major lipids (determined by measurement of

phospholipid phosphorus) [14]. Based on biochemical studies using detergent extraction and $(NH_4)_2SO_4$ fractionation procedures it has been suggested that most of the phosphatidylethanolamine and phosphatidylcholine is only loosely bound, whereas cardiolipin is tightly bound to the enzyme [14–17]. In particular, the removal of the last 2 or 3 cardiolipin molecules has been claimed to be very difficult [17,18]. The tightly bound cardiolipin has further been implied as the binding site for cytochrome *c* [1]. In contrast to the above studies on cytochrome *c* oxidase from bovine heart, cardiolipin can be removed entirely from yeast cytochrome *c* oxidase by multiple exchanges with phosphatidylcholine [19].

In previous studies the quantification of the individual lipids was accomplished by extracting the phospholipids with organic solvents followed by two-dimensional thin-layer chromatography. The phospholipids were then charred with H_2SO_4 and analyzed for inorganic phosphate. Here we suggest that high-resolution ^{31}P-nuclear magnetic resonance (NMR) provides a much more convenient access to the analysis of the lipid composition. We demonstrate that the individual lipid classes are spectroscopically visible in lipid-rich and lipid-depleted cytochrome *c* oxidase preparations without further biochemical manipulations. In addition, solid-state ^{31}P-NMR provides immediate insight into the molecular organisation of the phospholipids around cytochrome *c* oxidase.

Materials and Methods

Preparation of cytochrome c oxidase

Lipid-rich and lipid-depleted cytochrome *c* oxidase were prepared following the procedure of King and coworkers [15,16], with some modifications as detailed elsewhere [13,20,21]. In particular, 50 mM Tris-HCl (pH 7.4) was used instead of phosphate buffer in order to simplify the ^{31}P-NMR spectra. Table I summarizes the essential biochemical parameters of the lipid-rich and lipid-depleted samples measured in this work. Typical enzymatic activities are discussed in Ref. [13].

Lipid-rich cytochrome *c* oxidase and sample I of lipid-depleted cytochrome *c* oxidase were prepared in this laboratory according to Yu et al. [15]. Sample II was a gift from Prof. A. Azzi, Bern, delipidated also according to Yu et al. [15,20]. Sample III was prepared as sample I and subsequently further delipidated by purification on an affinity column with covalently bound cytochrome *c* in the presence of 0.1% Triton X-100 [21] (gift from Dr. L. Tamm). For the high-resolution ^{31}P-NMR experiments, the cytochrome *c* oxidase preparations were dissolved in 50 mM Tris-HCl buffer at pH 7.4 containing 1 wt.% sodium cholate. Since ^2H_2O was needed as a lock signal for the stabilization of the magnetic field, the buffer was made up

TABLE I

CHARACTERISTICS OF PHOSPHOLIPID-RICH AND PHOSPHOLIPID-DEPLETED CYTOCHROME *c* OXIDASE FROM BOVINE HEART

PL, phospholipid; cyt. ox, cytochrome *c* oxidase.

Protein preparation	Purity		Lipid content	
	nmol heme a/ mg protein	μg P_i/ mg protein	μg PL/ mg protein	mol P_i/ mol cyt. ox.
Lipid-rich preparation	4.9	9.09	220 [a]	140
Lipid-depleted preparations				
Sample I	8.5	2.6 [b]	63 [c]	20
Sample II [d]	7.8	1.69 [b]	41 [c]	14
Sample III [e]	10.7	1.24 [b]	30 [c]	7.5

[a] Literature: 200–250 μg PL/mg protein [16]; approx. 280 μg PL/mg protein [14].
[b] Literature: 1.5–1.8 μg P_i/mg protein [14].
[c] Literature: 59–25 μg PL/mg protein [16].
[d] Sample was gift from Prof. A. Azzi, Berne.
[e] Purified by affinity chromatography [21]; sample was gift from Dr. L. Tamm.

from a $H_2O/^2H_2O$ mixture (70:30, v/v). A 10 mm NMR sample tube was used containing approx. 1.5 ml solution with a total of 15–20 mg protein.

Solid-state ^{31}P-NMR measurements were made with densely packed pellets of cytochrome *c* oxidase preparations. To this purpose, the protein stock solution (containing buffer and some cholate) was diluted by about a factor of 200 with cholate-free buffer and centrifuged at $100\,000 \times g$ for 30 min. [13]. The tight pellet (containing about 70–80 wt.% water) was transferred into a NMR sample tube. About 100 mg protein was used for each spectrum, containing about 22 mg and 6 mg phospholipid for lipid-rich and lipid-depleted cytochrome *c* oxidase, respectively. Cardiolipin (from *Escherichia coli*), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were purchased from Avanti Polar Lipids, Birmingham, AL, U.S.A.

Magnetic resonance measurements

All NMR measurements were carried out with a Bruker-Spectrospin CXP-300 spectrometer with a ^{31}P frequency of 121.5 MHz. High-resolution ^{31}P-NMR spectra were obtained with a pulse width of 12 μs ($\pi/4$ pulse), a relaxation delay of 2.1 s, and a spectral width of 1 kHz. Phosphorus T_1 relaxation times of phospholipids in bilayers with and without protein are usually about 1–1.5 s [13,22,23]; the conditions specified above are hence sufficient to avoid saturation. Most high-resolution spectra were recorded without proton-decoupling. Low-power (5 W), broad-band decoupling reduced the linewidth of the phospholipids by about 4 Hz, but did not affect the linewidth of the inorganic phosphate which was typically 4 Hz (linewidth at half-height of resonance).

Solid-state ^{31}P-NMR spectra of wet pellets of cytochrome *c* oxidase were recorded with pulses of 4 μs ($\pi/2$ pulse) and inverse-gated proton decoupling (approx. 150 W). The spectral width was 31.25 kHz in this case.

Results and Discussion

Fig. 1 shows solid-state ^{31}P-NMR spectra of lipid-rich (A) and lipid-depleted (B) cytochrome *c* oxidase preparations and of a phospholipid model membrane (C) composed of phosphatidylcholine (POPC; 41 wt.%), phosphatidylethanolamine (POPE; 38 wt.%), and cardiolipin (isolated from *E. coli*; 21 wt.%). The model membrane gives rise to an axially symmetric shielding tensor, and the residual chemical shift anisotropy is $\Delta\sigma = -45$ ppm, as measured from the edges of the spectrum [24]. The spectrum of the lipid-rich cytochrome *c* oxidase which has approximately the same lipid composition as the model membrane (cf. below) is characterized by a quite similar shape with $\Delta\sigma = -44$ ppm. The lipid-to-protein ratio of the enzyme preparation is about 140 (mol P_i/mol cytochrome *c* oxidase) and Fig. 1A demonstrates convincingly that most lipids ($\geqslant 80\%$) are organized in a fluid-like bilayer structure. In contrast, the lipid-depleted cytochrome *c* oxidase preparation is char-

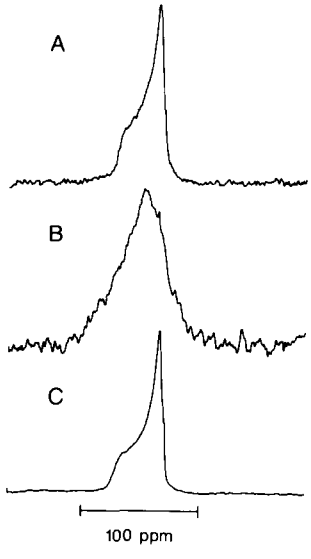

Fig. 1. Solid-state ^{31}P-NMR spectra of (A) lipid-rich cytochrome *c* oxidase (B) lipid-depleted cytochrome *c* oxidase and (C) multilamellar liposomes composed of cardiolipin (from *E. coli*; 21 wt.%), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (38 wt.%) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (41 wt%). Measuring temperature 15°C. Total spectral width 31.25 kHz. 150 W proton-decoupling power. Experimental conditions: (A) 100 mg lipid-rich cytochrome *c* oxidase containing 22 mg phospholipid. Measuring time, approx. 15 min; 450 scans. (B) 100 mg lipid-depleted cytochrome *c* oxidase containing 6 mg phospholipid. Measuring time, 14 h. (C) 30 mg phospholipid suspended in about 30 mg buffer. Measuring time, 5 min; 150 scans. The inflection at the high-field edge of the spectrum is due to the smaller chemical shift anisotropy of cardiolipin.

acterized by a different lineshape. The spectrum is broader and no longer typical for an axially symmetric shielding tensor. Due to the low lipid content (lipid-to-protein ratio of about 20 mol P_i/mol cytochrome *c* oxidase) the signal-to-noise ratio is low and an exact lineshape analysis is not possible. However, the spectrum allows the qualitative conclusion that the lipids are not completely immobilized since the total width of the spectrum ($|\Delta\sigma| \approx$ 90 ppm) is much narrower than that observed for rigidly fixed phospholipids in solid crystals ($|\Delta\sigma| \approx 180$ ppm) [25–28]. Motional narrowing of the ^{31}P-NMR spectra could originate from two different molecular mechanisms:

(1) a rotation of the individual phospholipid molecules on the protein surface and/or

(2) a rotation of the lipid with the protein as a whole. The latter possibility is, however, rather improbable in view of the dense packing of the pellet after high-speed ultracentrifugation.

It should be noted that based on the geometry of the enzyme at least 40 lipid molecules are necessary to form a continuous bilayer shell around cytochrome *c* oxidase [2]. The delipidated enzyme contains less than 20 lipid molecules, which is distinctly below this limit; a full lipid annulus can therefore not exist under these conditions.

Solid-state ^{31}P-NMR measures the envelope of all phospholipid resonances, and hence does not allow the identification of individual lipids. This, can however, be achieved by measuring the high-resolution ^{31}P-NMR spectra as shown in Fig. 2 for lipid-rich and in Fig. 3 for lipid-depleted cytochrome *c* oxidase. The spectra were obtained from the same biochemical preparations as used for the solid-state NMR spectra. However, in order to produce an optically clear solution, the protein was solubilized in cholate-containing buffer (50 mM Tris-HCl (pH 7.4), 1 wt.% sodium cholate). A defined amount of inorganic phosphate was added as an internal standard to quantify the phospholipid resonances. Besides inorganic phosphate three further resonances are well-resolved for lipid-rich cytochrome *c* oxidase (Fig. 2A), which arise from cardiolipin (−2.2 ppm), phosphatidylethanolamine (−2.6 ppm), and phosphatidylcholine (−3.2 ppm). The chemical shifts are referred to P_i (pH 7.4) defined as 0 ppm. The assignments are based on a comparison with the literature [29,30] and

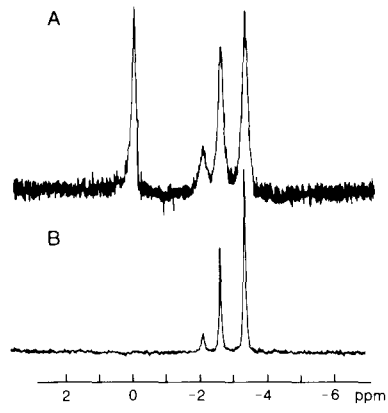

Fig. 2. High-resolution ^{31}P-NMR spectra (at 121.5 MHz) of (A) lipid-rich cytochrome *c* oxidase and (B) a mixture of cardiolipin (CL; 13 wt.%), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE, 39 wt.%)) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC; 48 wt.%). Measuring temperature, 5°C. 50 mM Tris-HCl buffer (pH 7.4) containing 1 wt.% cholate. Inorganic phosphate (P_i) added as an internal standard to lipid-rich cytochrome *c* oxidase. Spectral width, 1 kHz.

also with a mixture of synthetic lipids dissolved in the same buffer. The spectrum of the mixture composed of POPC (50 mol%), POPE (43 mol%) and cardiolipin (from *E. coli*, 7 mol%) is displayed in Fig. 2B. Integration of the spectra allows a quantification of the individual lipids. The average

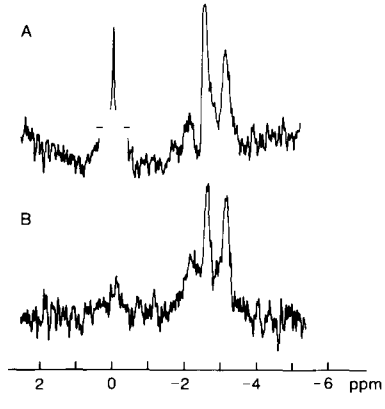

Fig. 3. High-resolution ^{31}P-NMR spectra (at 121.5 MHz) of lipid-depleted cytochrome *c* oxidase in 50 mM Tris-HCl buffer (pH 7.4) containing 1% cholate. Measuring temperature 5°C. (A) 14 mg protein with 0.57 mg lipid. Measuring time, 10 h. Sample was a gift from Prof. A. Azzi, Berne. The intense low-field peak originates from inorganic phosphate buffer, which was not completely removed. (B) 13.9 mg protein with 0.87 mg lipid. Measuring time, 11 h.

lipid composition of lipid-rich cytochrome *c* oxidase was found to be 50 mol% phosphatidylcholine, 41 mol% phosphatidylethanolamine and 9 mol% cardiolipin (average of 3 different spectra). These findings are consistent with the biochemical analysis as published previously [14,15]. The biochemical analysis also indicates traces of other phospholipids. These could not be detected by ^{31}P-NMR, probably because of too low signal intensity.

Fig. 3 compares two different preparations of the lipid-depleted enzyme. For both samples the signal-to-noise ratio is rather low due to the low total lipid content. Nevertheless, it is obvious that the resonance positions remain unchanged compared to lipid-rich cytochrome *c* oxidase. Furthermore it is again possible to quantify the percentage of the three major lipids (Table II).

The major difference between Figs. 2 and 3 is the intensity ratio of the individual lipids. While phosphatidylcholine is clearly the predominant lipid in lipid-rich cytochrome *c* oxidase, phosphatidylethanolamine and phosphatidylcholine occur in almost equal amounts in the lipid-depleted enzyme. The various spectra of Fig. 3 also indicate that there is a considerable variation in the lipid composition of the lipid-depleted enzyme depending on the extent of delipidation. In contrast to earlier conclusions that only cardiolipin remains associated with lipid-depleted cytochrome *c* oxidase [14,15,17,18], it follows from the NMR spectra that also appreciable amounts of phosphatidylcholine and phosphatidylethanol-amine remain bound. In fact, cardiolipin is the least abundant of the three lipid classes, even though there is a relative enrichment of cardiolipin compared to the lipid-rich preparation. In the most delipidated samples, phosphatidylethanolamine appears to be the predominant single lipid.

Finally, let us briefly consider the linewidth of the high-resolution ^{31}P-NMR spectra, since this is relevant to the question whether there exist 'NMR-invisible'-phospholipids. Cytochrome *c* oxidase can be approximated by a rotation ellipsoid of 110 Å in length and 60 Å in width [31,32]. The principal components of the axially symmetric diffusion tensor are then calculated as [33]:

$$D_{\parallel} = 4 \cdot 10^{6}\ \mathrm{s}^{-1} \quad D_{\perp} = 2.4 \cdot 10^{6}\ \mathrm{s}^{-1}$$

assuming a viscosity of 0.01 Poise. The corresponding correlation times are given by

$$\tau_{\perp} = (6D_{\perp})^{-1} = 7 \cdot 10^{-8}\ \mathrm{s}$$

$$\tau_{\parallel} = (2D_{\perp} + 4D_{\parallel})^{-1} = 4.8 \cdot 10^{-8}\ \mathrm{s}$$

Since the difference in the two correlation times is small, the calculations can be simplified by assuming a single correlation time of $\tau_c = 7 \cdot 10^{-8}$ s. The two relaxation mechanisms which determine the linewidth are

(1) chemical shift relaxation and

(2) dipole-dipole relaxation. In order to calculate the contribution of these two processes we consider an extreme case in which the lipids are rigidly bound to the protein so that the linewidth is determined exclusively by the protein motion

TABLE II

^{31}P-NMR ANALYSIS OF PHOSPHOLIPID-RICH AND PHOSPHOLIPID-DEPLETED CYTOCHROME *c* OXIDASE FROM BOVINE HEART

For abbreviations, see legend to Fig. 2.

Protein preparation	PC (mol%)	PE (mol%)	CL (mol%)
Lipid-rich preparation [a]	50 ± 1 [b]	41 ± 1	9 ± 1
Lipid-depleted preparations			
Sample I [a]	47	41	12
Sample II	28	58	14
Sample III	31	52	17

[a] The same preparation was also used for solid-state ^{31}P-NMR.

[b] Average of three measurements.

with a correlation time of $7 \cdot 10^{-8}$ s. Standard linewidth formulas for isotropic tumbling can be applied [34] and the contributions to the linewidth via chemical shielding anisotropy and dipole-dipole relaxation are estimated as 11 and 13 Hz, respectively. These calculations assume relaxation amplitudes of $|\Delta\sigma| = 90$ ppm and $|F_0|^2 = 3 \cdot 10^9$ s^{-2} for chemical shift and dipole-dipole relaxation, respectively, which are conservative estimates [22,23]. As an important consequence it follows that protein tumbling is sufficiently fast to give rise to sharp ^{31}P-NMR resonances even when the lipids are fixed rigidly to the protein surface. Hence the possibility of NMR-silent phospholipids can be excluded and the spectra shown in Figs. 2 and 3 truly reflect the correct ratio of the three major phospholipids.

In conclusion, high-field ^{31}P-NMR provides a simple and convenient means for the identification and quantification of the phospholipids copurified with cytochrome *c* oxidase. For the lipid-rich enzyme preparation the NMR analysis and the biochemical analysis yield virtually identical results. Diverging conclusions are however reached for lipid-depleted cytochrome *c* oxidase. While most previous biochemical studies claim that cardiolipin is the only remaining lipid the NMR data show that all three lipids can be observed with phosphatidylethanolamine being the predominant lipid species.

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