Biochimica et Biophysica Acta, 684 (1982) 47–52
Elsevier Biomedical Press

BBA 71020

^{31}P-NMR STUDIES ON PHOSPHOLIPID STRUCTURE IN MEMBRANES OF INTACT, FUNCTIONALLY-ACTIVE, RAT LIVER MITOCHONDRIA

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(Received August 12th, 1981)

Key words: ^{31}P-NMR; Phospholipid structure; Mitochondrial membrane; Oxidative phosphorylation; (Rat liver)

^{31}P-NMR studies of intact functional rat liver mitochondria at 37°C demonstrate that the large majority (≥95%) of endogenous phospholipids exhibit motional properties consistent with bilayer structure. This property is unaffected by oxidative phosphorylation processes or the presence of Ca^{2+}.

The inner mitochondrial membrane is the primary side of ATP synthesis in eukaryotic cells. The biochemistry of such oxidative phosphorylation has been investigated in great detail. However, very little is known about the molecular structure of this membrane particularly under functional conditions. Calorimetric and freeze-fracture studies suggest the occurrence of areas of liquid-crystalline bilayers with the overall morphology being dependent upon the functional state of the mitochondrion [1], although alternative interpretations of the freeze-fracture results are possible [2]. A major difficulty in obtaining reliable structural information in this system is that the mitochondrion is extremely labile. Most structure probing techniques require long data accumulation times or potentially harmful sample preparation procedures after which mitochondria are no longer active. This difficulty can (partially) be overcome by the use of ^{31}P-NMR, which can give quantitative information on the structure of phospholipids in membranes [3]. In our previous ^{31}P-NMR study on rat liver mitochondria [4] we have demonstrated that the biochemical (and possible structural) stability of an anaerobic mitochondrial suspension at 37°C was less than 5 min, shorter than the time needed to record the NMR spectrum. Therefore, less labile membrane derivatives like inner membrane ghosts were examined. It was found that at 37°C the phospholipids were mainly organized in bilayers with the possible exception of a small fraction of the phospholipids which underwent isotropic motion at the NMR time scale. This fraction was increased upon addition of Ca^{2+} [4].

It is clearly of interest to determine whether phospholipid experiencing such isotropic motion (which may indicate the presence of non-bilayer structures [3,4]) also occur in functional mitochondria. We report in this paper ^{31}P-NMR and saturation transfer ^{31}P-NMR [5,6] on mitochondria which are maintained in a functional state throughout the signal accumulation. This is achieved by using large (8 ml) sample volumes and by keeping the mitochondria well oxygenated. It is shown that under various conditions the membrane phospholipids give rise to ^{31}P-NMR spectra which are typical of extended bilayers

Rat liver mitochondria were isolated in 0.28 M sucrose, 1 mM Tris/HCl (pH 7.2) buffer as described in Ref. [7]. For NMR experiments the final mitochondrial pellet (typically 5 ml pellet

from ten male 150–200 g Wistar rats) was gently homogenized with the aid of a loose fitting teflon homogenizer in one volume of 0.25 M sucrose, 5 mM $MgCl_2$, 20 mM KCl, 20 mM Tris/HCl (pH 7.4) buffer (containing 20% ^2H_2O) and stored at 0°C for a maximum of 4 h. Using standard oxygraph techniques these mitochondria exhibited respiratory control ratios of 4–6 at 25°C. ^{31}P-NMR measurements were performed at 81 MHz on 8 ml mitochondrial samples (60–80 mg protein/ml) in 20 mm diameter tubes using a 25 kHz sweepwidth (4 K data points) and gated high power proton decoupling (10 watt input power during the acquisition time) as described before [8]. Under these conditions the ^{31}P-NMR spectrum of phospholipids organized in extended bilayers has the characteristic asymmetrical line shape with a low field shoulder and a high field peak [8,16]. The T_1 of the various resonances in the mitochondrial suspension at 0°C was determined with the inversion recovery method and was found to range from 0.1 to 0.2 s dependent on the resonance. These relatively short values probably are caused by small amounts of Mn^{2+} present in the mitochondria [17]. Since the employed inter-pulse time is long as compared to T_1, no significant saturation of resonances can occur. Furthermore, although due to the experimental set up we are unable to determine the absolute amount of signal in the spectrum other studies using several different membranes have demonstrated that in general the total signal is observed [18–20]. Saturation transfer ^{31}P-NMR experiments were done using the DANTE pulse technique [5,6]. Chemical shifts are reported with respect to the resonance position of phospholipids undergoing rapid isotropic motion (e.g. sonicated egg phosphatidylcholine vesicles). During the NMR experiment the mitochondrial suspension was stirred using an air driven propellor. Substrates could be injected into the sample in situ in about 10 s via an external syringe attached to an 1 mm inner diameter gauge connected to teflon tubes (1 mm inner diameter) running to the bottom of the NMR tube. Each substrate addition was followed by injecting twice 0.5 ml of the buffer thereby quantitatively delivering the substrate to the suspension. Complete mixing of the mitochondria with the injected substrates occurred in 30 s or less. The sample was kept oxygenated as required by pumping (at a rate of 40 μl/min) a 30% H_2O_2 solution into the mitochondrial suspension [9]. This suspension contained 0.1 ml of a 20 mg/ml catalase (Sigma, St. Louis) solution. For other NMR and experimental details see the figure legends. Protein was determined following Lowry et al. [10]. Small unilamellar vesicles of egg phosphatidylcholine were prepared by ultra-sonication. Chemicals were of analytical grade.

In Fig. 1A the ^{31}P-NMR spectrum of rat liver mitochondria at 0°C in state 4 (e.g. in the presence of succinate and oxygen, but in the absence of added nucleotides) is shown. This spectrum, which was obtained in only 3.3 min, is composed of an asymmetrical broad resonance typical for phospholipids organized in extended bilayers [3] and much narrower resonances arising from water soluble phosphates. These peaks can be assigned using the ^{31}P-NMR data obtained by Ogawa et al. [9] in the same system. The major narrow peak at -3 ppm is from internal P_i. The peak at -4.7 ppm wich shows up as a shoulder on the downfield side of the phosphate peak is a composite peak arising from phosphomonoesters such as glycero-3-phosphate, AMP, phosphocholine and the 2′-phosphates of $NADP^+$ and NADPH. The peak at $+4.7$ ppm corresponds to the chemical shift position of the γ-phosphates of ADP and ATP, whereas in the 8–10 ppm range a broad peak of the α-phosphates of ADP and ATP as well as the diphosphate peaks of NAD and NADP are present. This spectral region coincides with the high field peak of the phospholipid spectrum. At 18 ppm a small resonance of the β peak of ATP is present. This NMR spectrum was stable for at least 30 min. In the absence of succinate, catalase or oxygen very similar spectra were obtained in this time frame.

Saturation transfer ^{31}P-NMR is a useful technique to (partially) separate the NMR signals of the phospholipids from that of the water soluble phosphates [6]. Applying a saturation pulse train of 1 s at the position indicated by the arrow in Fig. 1A results in a strong reduction of the broad phospholipid ^{31}P-NMR signal (fig. 1B). The difference spectrum shown in Fig. 1C is almost entirely composed of the typical 'bilayer' signal of the phospholipids. The effective chemical shift ani-

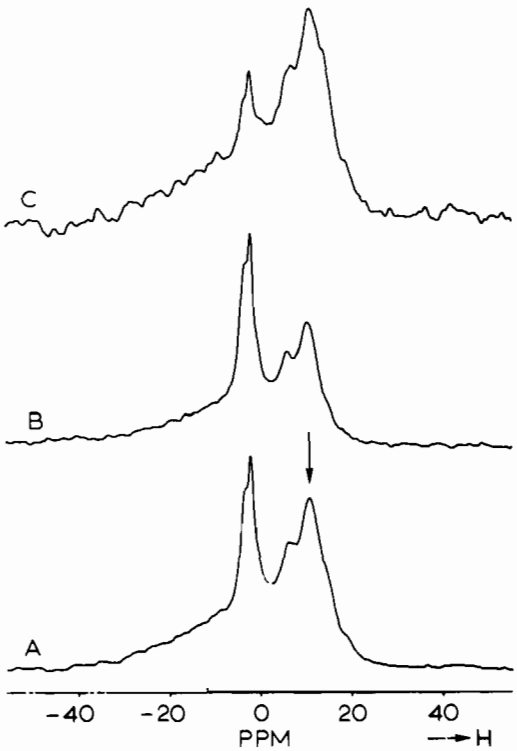

Fig. 1. Saturation transfer ^{31}P-NMR of intact rat liver mitochondria at 0°C. To 8 ml mitochondrial suspension (75 mg protein/ml) 2 ml 82.5 mM sodium succinate was added whereafter the solution was stirred and kept oxygenated as described in the experimental section. (A) Conventional ^{31}P-NMR spectrum obtained from 200 transients using 27 μs 90° radio frequency (r.f.) pulses and an inter-pulse time of 1 s. Delay time between pulse and data acquisition was 10 μs. (B) ^{31}P-NMR spectrum after saturation at the position indicated by the arrow in A. For saturation the DANTE pulse sequences of Morris and Freeman [5] as applied in membrane systems [6] was used. This sequence is: $|D_0\text{-}(P_1\text{-}D_1)_{NP}\text{-}P_1\text{-}D_2\text{-PW-De-acquisition}|_{NS}$. D_0 and D_2 are variable delays, P_1 and D_1 are the pulse width and delay between the saturation pulses. This sequence is repeated Np times, PW is the 90° r.f. pulse used for data acquisition. De is the 10 μs delay between PW and the data acquisition and NS is the number of times the whole sequence is repeated. In this experiment: $D_0 = 0$, $P_1 = 0.4$ μs, $D_1 = 100$ μs, Np = 10000, $D_2 = 10$ μs, PW = 27 μs, NS = 200. (C) Difference spectrum A − B. All free induction decays were exponentially filtered resulting in a 100 Hz line broadening. Spectra are plotted with a constant height of the main peak.

sotropy, which is a measure of the local order in the phosphate region of the phospholipids [11] can be estimated from this spectrum to be 38 ppm, which is typical for liquid-crystalline bilayers of phospholipids (including those from the inner mitochondrial membrane at this temperature [4,11]. The elimination of a small amount of the signal of the water soluble phosphates is due to some non-specific saturation, whereas the incomplete saturation of the 'bilayer' signal (Fig. 1B) is due to the lack of saturation power both being determined by experimental limitations of the spectrometer.

From the absence of a significant phospholipid resonance at the 0 ppm position (corresponding to phospholipids undergoing isotropic motion) and the −6 ppm position (which corresponds to the low field peak of the ^{31}P-NMR spectrum of mitochondrial phospholipids in the hexagonal H_{II} phase [4]), it can be concluded that the large majority (> 98% as estimated from Fig. 1C) of the phospholipids contributing to the ^{31}P-NMR spectrum have motional properties consistent with a bilayer organization of the lipids.

The ^{31}P-NMR spectrum (total data accumulation time 1.6 min) of a mitochondrial suspension in state 4 at 37°C is mainly composed of the same peaks of water soluble phosphates as observed in the spectrum at 0°C (Fig. 2A) This spectrum remains unchanged for another 1.6 min (Fig. 2B), but rapidly changes with longer incubation times. The main change being the growth and broadening of the phosphate peak as has been observed previously for mitochondria under anaerobic conditions [4].

Respiratory control ratios of the mitochondria before the start of the experiment and after recording the spectrum in Fig. 2B were unchanged at 4.0. Longer incubation times resulted in a rapid loss of respiratory control as found before [4]. Combining spectrum 2A with 2B and plotting the spectrum in a way such that the broad phospholipid signal is more apparent, shows that it has characteristic 'bilayer' line shape (Fig. 3A). From this spectrum and the absence of any significant peaks at 0 and −6 ppm we conclude that at 37°C mitochondria in state 4 also virtually all the phospholipids have motional properties characteristic of lipid bilayes. Since the saturation transfer NMR measurements require data accumulation times which were long as compared to the stability of the mitochondria at 37°C no additional information could be obtained by this technique.

The mitochondria were brought into active oxidative phosphorylation (state 3) by injecting

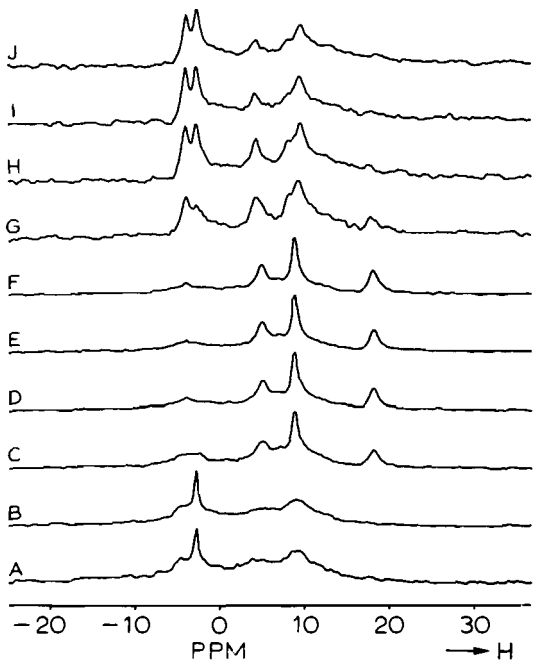

Fig. 2. ^{31}P-NMR of rat liver mitochondria at 37°C under conditions of oxidative phosphorylation and Ca^{2+} uptake. To 8 ml mitochondrial suspension (75 mg protein/ml), 2 ml 82.5 mM sodium succinate was added whereafter the solution still at 0°C was stirred and kept oxygenated as described in the experimental section. The sample was warmed to 37°C in 60 s by placing the tube in a 40°C water bath whereafter the tube was immediately placed in the NMR probe at 37°C and the automated data accumulation was started. Spectrum A was obtained from 100 transients using 27 μs 90° r.f. pulses with an 1-s inter-pulse time. After the accumulation 10 s was waited to allow injection of substrates after which the next accumulation was started. Under identical conditions at the end of experiment B and F 1 ml (14 mg/ml) ADP and 1 ml 1 M $CaCl_2$ were injected, respectively. To allow better visualization of the sharp peaks of the water soluble phosphates over that of the broad peak of the phospholipids, a line broadening of only 20 Hz was applied to the free induction decays.

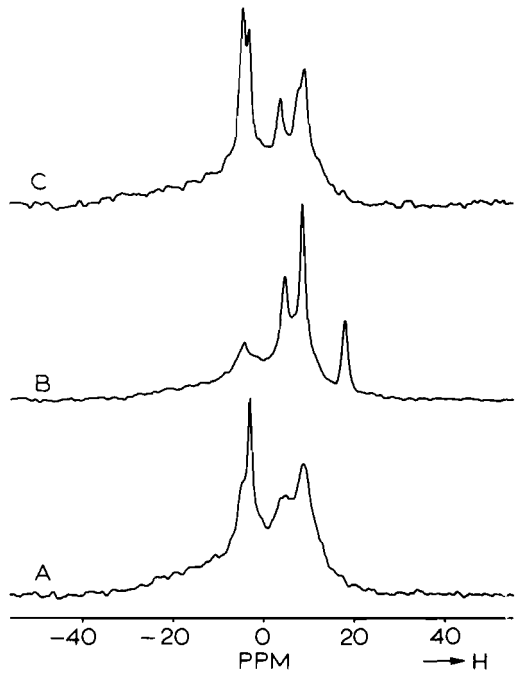

Fig. 3. ^{31}P-NMR of rat liver mitochondria at 37°C under conditions of oxidative phosphorylation and Ca^{2+} uptake. For experimental details see the legend of Fig. 2. (A) Combined spectrum of Fig. 2A and B. (B) Combined spectrum of Fig. 2C, D, E and F. (C) Combined spectrum of Fig. 2G, H, I and J. To allow better visualization of the broad phospholipid spectrum a line broadening of 50 Hz was applied to the combined free induction decays.

ADP into the suspension. ATP synthesis occurred immediately as could be detected from the strong decrease in signal intensity of the phosphate peak and the appearance of the β-phosphate peak of ATP (Fig. 2). No phosphate was added to the mitochondria so that they were forced to use their endogenous phosphate for ADP phosphorylation. This removed the strong free phosphate peak which interferes with the observation of possible isotropic phospholipid signals. The ^{31}P-NMR spectra of the mitochondria remained unchanged for at least 10 minutes (data from the first 7 min are shown in Figs. 2C to F); whereafter the phosphate peak increased again in intensity and the ATP signals disappeared. This was not due to substrate limitations as excess O_2 and succinate were present in the suspension. In the absence of oxygen no ATP synthesis occurred. In this case ADP addition resulted in an immediate hydrolysis of the ADP as detected from an increase in the phosphate peak and the peak at −4.7 ppm (chemical shift of AMP). No ADP or ATP signals could be detected under these conditions. From the total absence of an isotropic phospholipid peak in the spectra 2C to 2F and the shape of the phospholipid signal in the combined spectrum as shown in Fig. 3B it can be concluded that also during active oxidative phosphorylation the great majority (>95%, as estimated from Fig. 3B) of the phospholipids

maintain motional characteristics typical for extended bilayers.

Ca^{2+} is an uncoupler of oxidative phosphorylation and is rapidly taken up by mitochondria [12,13]. As Ca^{2+} induces non-bilayer lipid structures in the isolated mitochondrial inner membrane lipids and induces rapid isotropic motion of part of the lipids in isolated inner membranes [4] it is of interest to establish the effect of Ca^{2+} on the phospholipid structure in functionally intact mitochondria. Incubating the mitochondrial suspension with 0.07 M $CaCl_2$ (which is sufficient to cause large changes in the ^{31}P-NMR spectra of the phospholipids in isolated membranes and derived liposomes [4]) resulted in an immediate (within 4 min) disappearance of the ATP signals (Figs. 2G–F) and the appearance of the phosphate peak and a peak at −4.7 ppm which most likely originates from AMP [9], clearly demonstrating the uncoupling action of Ca^{2+}. Further incubation with Ca^{2+} resulted in rapid and complex changes in the NMR signals from the water soluble phosphates which at present are not understood. The important finding from the membrane structure point of view is that in the presence of this high concentration of Ca^{2+} the phospholipid signal is not significantly affected and that neither isotropic nor hexagonal H_{II} type of phospholipid ^{31}P-NMR signals are observed demonstrating maintenance of bilayer structure of the majority of the phospholipids under conditions in which there is a rapid Ca^{2+} transport across the membrane [12,13]. Very similar results were obtained when corresponding experiments were carried out at 25°C.

The present study demonstrates that it is possible to obtain by ^{31}P-NMR structural information on the membrane phospholipids of rat liver mitochondria and to follow the structure during functioning of the mitochondria. It is shown that in the 0–37°C temperature range both in the resting state in the presence of Ca^{2+} as well as during oxidative phosphorylation the majority (> 95%) of the phospholipids give rise to ^{31}P-NMR signals typical of phospholipids organized in extended liquid-crystalline bilayers. These findings have several implications for the structure of the lipid part of this membrane. The lipids of the inner mitochondrial membrane (40% phosphatidylcholine, 40% phosphatidylethanolamine and 20% cardiolipin [14]) like those of other biological membranes can be divided on structural grounds into two groups. These correspond to lipids preferring a bilayer organization such as phosphatidylcholine and cardiolipin in the absence of Ca^{2+} and those preferring the H_{II} organization such as phosphatidylethanolamine and cardiolipin in the presence of Ca^{2+} [4]. In the hydrated total lipid extract at 37°C the non-bilayer preference of the phosphatidylethanolamine is expressed by the appearance of an 'isotropic' phase which is associated with the presence of lipidic particles, likely of an inter bilayer inverted micellar origin. The amount of non-bilayer structures is greatly increased by the presence of Ca^{2+}. These observations together with our present findings suggest a strong bilayer stabilizing role of the inner mitochondrial membrane proteins. A similar bilayer stabilizing role has been suggested for rhodopsin in the photoreceptor membrane [15]. In this context it should be realized that the protein content of the mitochondrial inner membrane is extremely high (approx. 75% by weight). The bilayer stabilization is maintained during substrate oxidation, proton transport and phosphorylation of ADP. From our previous observations that at 37°C part of the phospholipids in isolated inner mitochondrial membranes undergo isotropic motion, which fraction was increased in the presence of Ca^{2+} [4] we can suggest that probably during isolation of the inner mitochondrial membrane structural changes (possible fragmentation) occur which lead to an increased phospholipid polar headgroup motion.

This work was supported by NATO Research Grant No. RG-209.80.

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