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TEMPERATURE-DEPENDENT LATERAL DIFFUSION OF PHOSPHOLIPIDS IN HEPATIC MICROSOMES AS STUDIED BY ^{31}P-NMR

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(1) The 81 MHz ^{31}P-NMR spectra of isolated rabbit liver microsomes before and after trypsin treatment and of the total microsomal lipid extract were recorded in the 4–40°C temperature range. (2) In both treated and untreated microsomes at 4°C most of the phospholipids gave rise to typical bilayer spectra whereas the lineshape of the latter in the 25–37°C temperature range becomes narrower and more symmetrical. (3) Quasi-elastic light scattering (QELS) measurements revealed that the microsomes maintain their size in the temperature region of the measurements. (4) We interpret the lineshape changes for untreated microsomes above 25°C as being determined by lateral diffusion. This is supported by lineshape calculations as a function of the lateral diffusion coefficient. (5) The different spectral behavior of enzymatically active (untreated) and inactive (treated) microsomes suggests that the membrane proteins influence the lateral diffusion of the phospholipids.

Introduction

The temperature dependence of ^{31}P-NMR of microsomes (small vesiculated fragments originating from the hepatic endoplasmic reticulum) is well documented [1–4]. But the prevalence of a narrow and more isotropic lineshape at 37°C is not well understood until now. Some authors explained it by the formation of intrabilayer reversed micelles, so called 'lipidic particles', in which the phospholipids undergo isotropic motional averaging on the NMR timescale [2]. But if a considerable proportion of the phospholipids (up to 60% according to Ref. 2) are transformed into a reversed micellar phase state the microsomes can not be stable. The reversibility of such a process by simply cooling down the sample is furthermore unlikely from the thermodynamical standpoint. In an other paper of De Kruijff et al. [1] the possibility that lateral diffusion of the phospholipids could be the main source of isotropic motional averaging in microsomes was discussed. Other authors demonstrated that the reconstitution of cytochrome *P*-450 gives rise also for isotropic motional averaging of a part of the phospholipids [3]. In this paper we present evidence that lateral diffusion is the main source of the isotropic motional averaging in microsomes by comparison of ^{31}P-NMR spectra at various temperatures with spectra calculated as a function of the lateral diffusion coefficient. Moreover, the influence of tryptic treatment of microsomes on the ^{31}P-NMR lineshape is studied.

Abbreviations: QELS, quasi-elastic light scattering; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid.

Materials and Methods

Liver microsomes were prepared from phenobarbital induced rabbits as described previously [5]. The final pellet was washed with 100 mM pyrophosphate buffer (pH 7.4) to remove ribosomes and after that four times with 50 mM Hepes buffer (pH 7.3) which contained 1 mM EDTA (buffer A) to remove the pyrophosphate and the loosened ribosomes. The final pellet was resuspended in buffer A which contained 10% ^2H_2O to a final concentration of 30–50 mg microsomal protein per ml (180–250 nmol cytochrome *P*-450 per ml). In samples of trypsin-treated microsomes the protein content was approx. 20% lower due to the protein loss by trypsinization (see below). The protein content was determined according to the method of Lowry et al. [6] with ovalbumin as standard, the cytochrome *P*-450 content according to Ref. 7. Part of the pyrophosphate washed microsomes was treated with trypsin. The trypsin was added (trypsin/microsomal protein = 1 : 50 by weight) and incubated at room temperature for 1 h under mild stirring. The protein content before and after trypsinization was 24 mg/ml and 18.6 mg/ml (corresponding to a protein loss of 22.5%). In the supernatant of the first washing step after trypsin treatment we determined a protein content of 6.0 mg per ml trypsinized microsomal dispersion. The phospholipid composition was not significantly changed by the treatment (Fig. 1). The treated microsomes were also four times washed and the pellet resuspended analogously to the control.

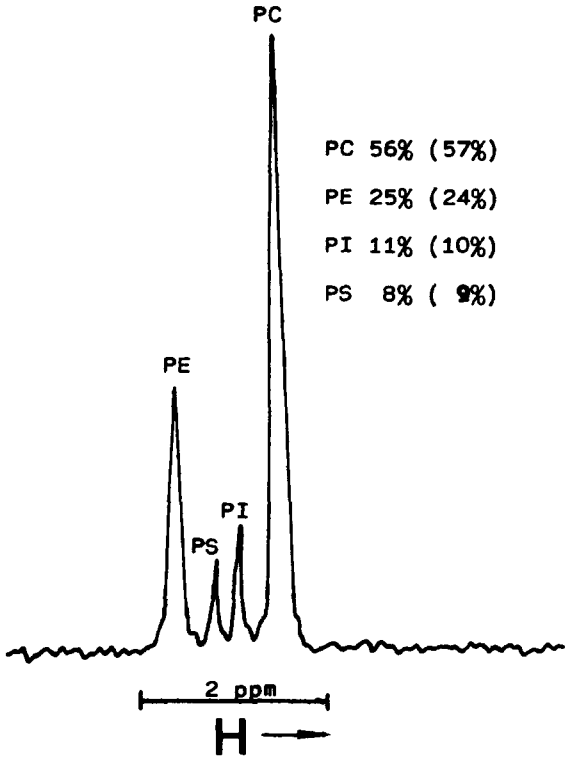

Fig. 1. 81 MHz ^{31}P-NMR spectrum of the total lipid extract of trypsin-treated microsomes in a 1:1 chloroform/methanol mixture and the phospholipid compositions (mol%) of untreated and treated ( ) microsomes. 3000 transients were accumulated for each spectrum with a 10 s interpulse time.

Microsomal lipids of untreated and treated microsomes were obtained by extraction according to Bligh and Dyer [9] and stored at −20°C in a 1 : 1 chloroform/methanol mixture. The lipids of untreated microsomes were dried from this solution under vacuum (4 h) and the lipid film was dispersed in buffer A containing 10% ^2H_2O at a concentration of 30 mg phospholipid per ml. The phospholipid compositions of treated and untreated microsomes were determined by ^{31}P-NMR in a 1 : 1 chloroform/methanol mixture containing 10% C^2HCl_3 (Fig. 1). For quantitation the single heights of the individual phospholipid bands were taken.

The ^{31}P-NMR measurements were performed at 81 MHz under the conditions of broad band inverse gated proton decoupling using a Bruker WP-200 spectrometer. We accumulated 500–1500 transients using 90° r.f. (radio frequency) pulses with a 2-s interpulse time if not indicated otherwise. The temperature was constant to within ± 1°C. For each temperature step above 15°C we used a fresh sample, heated up from its storage temperature (4°C) in a water bath, to prevent loss of enzymatic activity.

QELS measurements (for review see Ref. 10) were carried out in the homodyne autocorrelation mode using the apparatus described in Ref. 11 and an argon laser (488 nm). The square root of the measured correlation function was analyzed by the cumulant method [12]. The apparent diameters of the microsomes were derived from the first cumulant K_1 and $\delta_z = K_2/K_1^2$ (K_2 is the second cumu-

lant) can be used as a measure of the polydispersity of the sample.

The samples (concentration approx. 0.3 mg protein per ml) were cleaned by filtering the suspension through 1 μm Nuclepore filters directly into the rectangular scattering cells. The temperature maintenance was better than ± 0.1°C.

Electron microscopic measurements of untreated and treated microsomes were performed using the negative contrast method. The samples were fixed in glutaraldehyde at 4°C and contrasted with osmium tetroxide.

The ^{31}P-NMR lineshapes were calculated by means of a computer program as described previously [13]. The procedure is based on Anderson's stochastic theory of resonance linewidth [14] with a numerically evaluated relaxation function [15], assuming an axially symmetrical ^{31}P chemical shift tensor. This anisotropy is reduced by lateral diffusion of the phospholipids across the surface of a sphere. The diffusion is simulated by Monte Carlo jumps of single molecules with defined jumplength and correlation time. The spectra were calculated as a function of the lateral diffusion coefficient assuming $\Delta\sigma_{eff} = 43.2$ ppm and a 50 Hz Lorentzian line broadening.

Hepes and Tris buffer as well as trypsin were obtained from Serva (F.R.G.). The water used was bidistilled and ion-exchanged. All other reagents were of reagent grade and used without further purification.

Results and Discussion

^{31}P-NMR spectra of untreated microsomes are shown in Fig. 2B. A relatively narrow line grows at the position of the isotropic line with increasing temperature without the asymmetric lineshape being changed. Above 25°C a second effect becomes prevalent. The asymmetry of the 'bilayer' signal decreases and the high-field shoulder approaches the isotropic line position. The line changes to a narrow and more symmetrical one up to a temperature of 37°C. In order to exclude the possibility that this reversible behavior is caused by decreasing vesicle size with increasing temperature we determined the vesicle size of microsomes as a function of the temperature using QELS. As can be inferred from Table I the measurements give the evidence that the microsomes maintain their size in the temperature range investigated.

By use of electron microscopy number averaged diameters of untreated microsomes of 140 nm (80–200 nm) normally distributed in the indicated size region were evaluated. For comparison of the sizes of microsomes determined by the two meth-

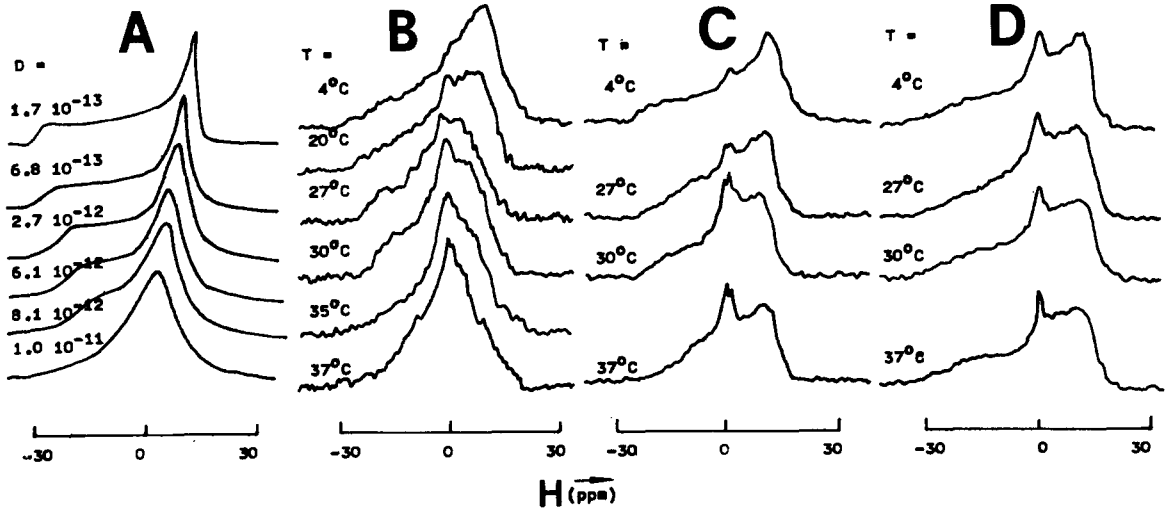

Fig. 2. 81 MHz ^{31}P-NMR spectra calculated as a function of the lateral diffusion coefficient D (m^2/s) as indicated (A) and 81 MHz ^{31}P-NMR spectra of untreated microsomes (B), trypsin-treated microsomes (C) and a dispersion of total microsomal phospholipids (D) at various temperatures as indicated. 50 Hz line broadenings were applied to the free induction decays.

TABLE I

APPARENT VESICLE DIAMETERS AT VARIOUS TEMPERATURES OF UNTREATED AND TREATED ( ) MICROSOMES AT A 90° SCATTERING ANGLE

Temperature (°C)	δ_z	d_{app} (nm)
5	0.15(0.10)	200(164)
20	0.13(0.10)	208(163)
37	0.11(0.11)	202(161)

ods one must take into account that QELS measures z-averages. In the case of polydisperse samples the apparent diameters are therefore larger than the number-averaged diameters.

The increase of the medium viscosity by addition of sucrose or glycerol up to 5-fold which would reduce the tumbling rate accordingly has no effect on the ^{31}P-NMR lineshape at 37°C in agreement with results of De Kruijff et al. [2]. Therefore we can exclude changes in vesicle size as well as tumbling effects as potential sources of isotropic motional averaging. The spectra calculated as a function of the lateral diffusion coefficient (assuming the vesicle diameter revealed by electron microscopy) are very similar to the spectra of untreated microsomes at the appropriate temperature (Fig. 1A, B). The lineshape of the former is very sensitive to changes of D in the region of $D = 10^{-12}$–10^{-11} m^2/s. The lateral diffusion coefficient of phospholipids in microsomes at 37°C was estimated to be 10^{-11} m^2/s by Galla et al. [19]. This is in good agreement with our results by comparison of measured and simulated spectra (Fig. 1A, B). To sum up all facts mentioned above and the good agreement of theoretical and experimental results is evidence that variations of the lateral diffusion coefficient of the phospholipids cause the lineshape changes of untreated microsomes above 25°C. This conclusion was also discussed, without providing evidence, by De Kruijff et al. [1].

In order to study the influence of proteins on the lateral diffusion we modified the microsomes by a treatment with trypsin. This protease can split those polypeptide chains of the protein which protrude the surface of the membrane. The treatment caused a slight decrease in size of the microsomes (Table I). The diameter of trypsinized microsomes determined by electron microscopy was 130 nm, normally distributed between 90 and 180 nm. We observed no changes in shape of the microsomes due to trypsin treatment using electron microscopy.

The ^{31}P-NMR spectra of trypsinized microsomes are shown in Fig. 1C. A relatively small and narrow line grows steadily with increasing temperature at the isotropic line position but the typical bilayer lineshape remained unchanged. Probably the narrow line is caused by the same effect as in the case of the untreated microsomes above 4°C. With the exception of the changing narrow line the temperature dependent behaviour of treated microsomes is similar to that observed in a dispersion of total microsomal lipids (Fig. 1D). On the other hand vesicles of pure dioleoylphosphatidylcholine with up to approximately 150 nm radius give symmetrical ^{31}P-NMR spectra which gets increasingly broader with increasing size as demonstrated in Ref. [21]. Apparently the proteins and the lipid composition influence the lateral diffusion coefficient of the phospholipids. Thus we have three different cases: In trypsinized microsomes the lateral diffusion is restricted so much that the lateral diffusion coefficient is below the sensitive region of D (10^{-12}–10^{-11} m^2/s) over the whole temperature range investigated whereas in PC vesicles the lateral diffusion coefficient is above the sensitive region so that in both cases no lineshape changes occur. In untreated microsomes the lateral diffusion coefficient is between that of the first two cases and changes over the sensitive region with increasing temperature.

It is unlikely that this behavior is caused by proteins (phosphorylated proteins or protein nucleic acid complexes) alone because it is a common finding that the lateral diffusion of the proteins is at least one magnitude slower than that of phospholipids. Moreover, the microsomes investigated were smooth due to the washing in pyrophosphate. This is also evidenced by the ^{31}P-NMR spectra of untreated microsomes at 4°C (Fig. 2B) which is free of a narrow isotropic line caused by ribosomes.

It is more likely that lipid-protein interactions are responsible for that behavior. One possible explanation is the gel-to-liquid phase transition of

the lipids of some protein lipid complexes (e.g. cytochrome *P*-450/PE as proposed in Ref. 4) as suggested by Stier et al. [16] and by Funk et al. [18] which might cause such an increase of the lateral diffusion of parts or of all lipids in the membrane of untreated microsomes.

Cytochrome *P*-450 is the main protein component of the microsomes (it may account for nearly 20% of the total membrane proteins by weight) and might be involved in this behaviour because it is inactivated after trypsinization by tryptic splitting of the hydrophilic part of cytochrome *P*-450 reductase. This inactivation by trypsin treatment might influence the phase transition in the membrane and in this way decrease the lateral diffusion coefficient of the phospholipids.

We can draw the following conclusions: (1) The ^{31}P-NMR lineshape of microsomes is determined by lateral diffusion. (2) The lateral diffusion coefficient of phospholipids in untreated microsomes changes over at least two magnitudes in the 4–37°C temperature range and is probably influenced by such proteins as can be split by trypsin treatment. The lateral diffusion coefficient of phospholipids in untreated microsomes is 10^{-11} m^2/s at 37°C whereas that in trypsin treated microsomes at the same temperature is below 10^{-12} m^2/s.

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