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Direct transition of dioleoylphosphatidylethanolamine from lamellar gel to inverted hexagonal phase caused by trehalose

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We have studied the effect of trehalose, a disaccharide of glucose, on the hydrated gel to liquid-crystalline and lamellar to inverted hexagonal phase transitions of dioleoylphosphatidylethanolamine using Fourier transform infrared spectroscopy, differential scanning calorimetry and X-ray diffraction. The data obtained by the first two methods suggested that the lamellar to hexagonal transition was eliminated in the presence of trehalose. However, the structural information derived from X-ray diffraction shows that the trehalose induces a direct transition from a lamellar gel phase to a liquid-crystalline inverted hexagonal phase without an intervening lamellar liquid-crystalline phase.

Introduction

This laboratory (Davis, CA) has recently suggested in two reviews [1,2] and two abstracts [3,4] that trehalose, a disaccharide of glucose, has the ability to stabilize hydrated phosphatidylethanolamines in the lamellar phase. Our original experiments, which used the techniques of differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR), suggested that trehalose might prevent H_{II} formation by hydrated phosphatidylethanolamines. We proposed that the trehalose might be able to do so by directly hydrogen-bonding to the phosphate and amino groups of the phosphatidylethanolamine, thereby preventing the hydrogen bonding of adjacent phosphatidylethanolamine headgroups which has been suggested to contribute to the tendency of these phospholipids to enter the inverted hexagonal (H_{II}) phase [5,6].

However, in our most recent study of the hydrated trehalose/phosphatidylethanolamine system we have added to DSC and FTIR the method of X-ray diffraction. The results demonstrate clearly that rather than preventing H_{II} phase, the presence of trehalose promotes the formation of the H_{II} phase. In doing so the H_{II} forms directly from the gel (L_{β}) phase without an intervening lamellar liquid-crystalline (L_{α}) phase. We present the evidence from all three experimental techniques in the present paper.

Materials and Methods

Sample preparation. L-α-Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and used without further purification. The DOPE was purchased in small lots and used immediately upon opening the vial. Occasional checks of DOPE with thin-layer chromatography showed only a single spot. Trehalose dihydrate was obtained from Pfanstiehl Laboratories (Waukegan, IL) and was placed under high vacuum overnight before storage in clean glass bottles. Trehalose solutions were prepared by dissolving the carbohydrate in distilled, deionized water to 1 M.

Samples were prepared according to the following procedure. DOPE and butylated hydroxytoluene (BHT) as an antioxidant were dissolved in chloroform and mixed to a ratio of 0.4 μg BHT/mg DOPE. The chloroform was evaporated under dry nitrogen and the sample was placed under high vacuum (10–20 mTorr) on a Virtis lyophilizer for at least 12 h. The lipid samples were rehydrated at room temperature (above the lamellar to H_{II} transition of DOPE) with either distilled

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water or a 1 M trehalose solution. The mixtures were temperature cycled at least three times between −20°C and room temperature with vortexing at room temperature during each cycle.

Fourier transform infrared spectroscopy (FTIR). The dried lipid samples were rehydrated as described above with either distilled, deionized water or the 1 M trehalose solution. The hydrated samples were placed between BaF_2 windows and mounted in an electric cooling cell (Peltier). The temperature was measured by a fine thermistor placed in contact with the BaF_2 window. Prior to recording spectra, samples were equilibrated at −15°C for at least 2 h. Spectra were recorded over a temperature range of −15°C to +40°C at 2 C° intervals. Samples were allowed to equilibrate for at least 10 min at each temperature before a new spectrum was recorded. FTIR was performed on a Perkin-Elmer 1750 spectrometer, and spectra were analyzed on a Perkin-Elmer 7500 laboratory computer. The wavenumber of the vibration of the CH_2 symmetric stretch of the acyl chains was found to the nearest 0.1 wavenumber using a 'center of gravity' algorithm [7].

Differential scanning calorimetry (DSC). The dried lipid samples were rehydrated with distilled, deionized water or trehalose solution and cycled as described above. A small amount of the mixture (5–20 mg lipid) was placed inside an airtight ampoule and calorimetric scans were recorded using a high sensitivity differential scanning calorimeter (Hart DSC 7707 Series, Hart Scientific, Provo, UT) over a temperature range of −15 to +40°C at a scan rate of 20 C°/h. Analysis was performed on an IBM-PC XT using Hart Scientific software.

X-ray diffraction. Samples were prepared as described for FTIR and DSC. Centrifuged pellets with a little added powdered teflon for calibration of X-ray spacings were mounted and sealed between mica sheets 1 mm apart in X-ray sample holders. Samples were prepared in Davis and sent to Canada on dry ice, or prepared in Canada and held for 24–48 h. There was no difference between the samples prepared in the two laboratories. The samples were held at various temperatures for at least one hour before a picture was taken. X-ray diffraction was carried out as previously described [8]. Multilamellar phases were identified by a series of reflections, all orders of a single dimension representing the repeat spacing, d. Hexagonal phases were identified by X-ray spacings all bearing ratios to the dimension of the first order, d_{hex}, of 1, $1/\sqrt{3}$, $1/\sqrt{4}$, $1/\sqrt{7}$, $1/\sqrt{9}$, etc., characteristic of a two-dimensional hexagonal network. Although X-ray data does not distinguish between H_I and H_{II}, we assume that this hexagonal phase is the inverted hexagonal or H_{II} phase, consistent with all previous data on phosphatidylethanolamines. Crystalline hydrocarbon chains, characteristic of the L_β phase, were seen as a sharp 4.1 Å high angle line in place of the broad 4.5 Å band seen for disordered chains characteristic of the L_α phase. Coexistence of lamellar and hexagonal phases was identified by the coexistence of two sets of reflections that index as described above, their relative amounts are given roughly by the intensities of those two populations of reflections. We report here the parameters d, the lamellar repeat distance that is made up of the sum of bilayer and interbilayer water layer thicknesses, and d_{hex}, where $2d_{hex}/\sqrt{3}$ is the distance between the axes of the water cylinders in the hexagonal structures.

Results

Fourier transform infrared spectroscopy

Both the gel to lamellar liquid-crystalline and lamellar to hexagonal phase transitions are characterized by increases in the number of *gauche* conformers in the hydrocarbon chains. These transitions have been studied in natural egg phosphatidylethanolamine (egg PE) using FTIR [9]. The change in vibrational frequency, expressed as wavenumbers (cm^{-1}) of the CH_2 symmetric stretch as a function of temperature has been used to provide information about the degree of disorder of the acyl chains and the position and nature of the thermotrophic phase transitions of the PE [9]. As egg PE undergoes the gel to liquid-crystalline phase transition, there is a sharp increase in the number of *gauche* conformers (or disorder) in the acyl chains as shown by the increase in wavenumber, with a second smaller increase in disorder at the lamellar to H_{II} transition temperature [9]. Similar transitions can also be seen in DOPE hydrated in pure water. A plot of the wavenumbers of the CH_2 symmetric stretch against temperature yields the data shown in Fig. 1a. The transition which starts around −8°C and is completed at +2°C correlates with the change from gel to liquid-crystalline phase. The smaller transition centered around +8°C correlates with the L_α to H_{II} transition. Also shown in Fig. 1a are data for changes in bandwidth with temperature, which provides an indication of the vibrational and rotational freedom of the CH_2 chains [10]. These data suggest that the increase in *gauche* conformers as the lipid undergoes the gel to liquid-crystalline and H_{II} transitions occurs in concert with changes in vibrational and rotational freedom for the CH_2 chains.

When the DOPE is hydrated in the presence of trehalose, only one phase transition is seen with FTIR: a rapid increase in wavenumber of the CH_2 symmetric stretch between −8°C and 0°C (Fig. 1b). This large increase in wavenumber is indicative of a transition from order to disorder, while the slow increase in wavenumber at temperatures above 0°C could be an indication of increasing number of *gauche* conformers with increasing temperature. However, in the presence of trehalose the bandwidth increases through the first tran-

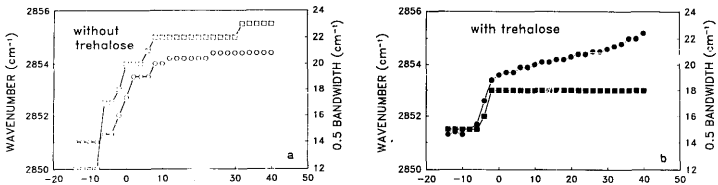

Fig. 1. (a) Temperature profiles showing the CH_2 symmetric stretching vibration at 2850 cm^{-1} (open circles) and the half bandwidth of the same vibration (open squares) for pure hydrated DOPE. (b) Temperature profiles for the CH_2 stretching vibration at 2859 cm^{-1} (closed circles) and the half-bandwidth (closed squares) for DOPE in 1 M trehalose.

sition (−8 to 0 °C) but shows no further increase even though the wavenumber of the CH_2 symmetric stretch increases. This result suggests that with higher temperatures in the presence of trehalose there is a slow increase in the number of *gauche* conformers but that the overall rate of molecular motion in the acyl chains is not increased.

Differential scanning calorimetry

Calorimetric scans of hydrated DOPE in the presence and absence of trehalose are shown in the DSC traces in Fig. 2. Pure hydrated DOPE has a sharp, symmetrical transition with a peak (T_m) at −8.6 °C with a large enthalpy. It was not possible to determine the onset or the true ΔH_{cal} (calorimetric enthalpy) of the transition because the samples could not be supercooled below −12 °C for the pure hydrated DOPE or −15 °C for the DOPE hydrated with trehalose solution. Upon further heating, the DOPE in water undergoes a smaller broad transition centered around +8 °C with a ΔH_{cal} of 1.25 kJ/mol, corresponding to literature values [11]. In the presence of trehalose, the hydrated DOPE showed no further calorimetric transitions following the large enthalpy gel to liquid-crystalline transition at about −8 °C.

X-ray diffraction

The results are summarized in Fig. 3, and representative X-ray photographs are shown in Fig. 4. For pure DOPE, the phases observed were strongly dependent on the thermal history of the samples, particularly on the time spent at each temperature. For times on the order of hours the phase transitions observed were consistent with the changes seen in FTIR and DSC. The phases determined from the X-ray reflections were as follows: L_β was seen below −8 °C, L_α between −8 °C and

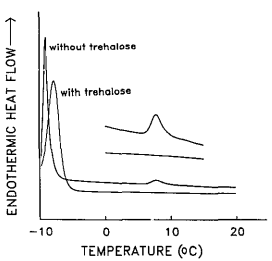

Fig. 2. Differential scanning calorimetry traces showing the transitions of DOPE in the presence of 1 M trehalose or water. There is an apparent small increase in the main transition temperature in the presence of the trehalose. The lamellar to H_{II} transition seen at about 8 °C in the presence of water is apparently absent in the presence of the trehalose. Inset: lamellar to H_{II} transition (top trace) in hydrated DOPE and the corresponding temperature region for DOPE in 1 M trehalose (bottom trace) expanded five times.

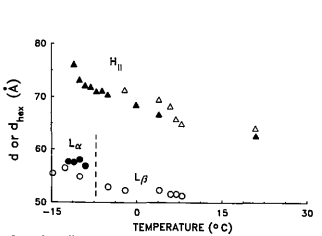

Fig. 3. a. Lamellar (circles) and hexagonal (triangles) spacings of DOPE in excess water (open symbols) or in 1 M trehalose (filled symbols) as determined by X-ray diffraction. The dashed vertical line demarcates, for the lamellar phases, the boundary between the crystalline and disordered configuration of the hydrocarbon chains. The X-ray diffraction results show that there is no lamellar liquid-crystalline (L_α) phase in DOPE in the presence of trehalose. Without trehalose, ice and a small quantity of a second phase coexist with the L_β lamellar phase.

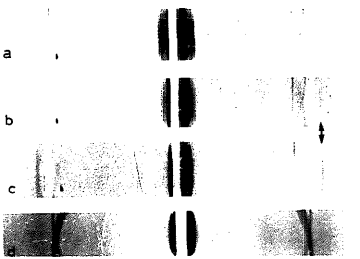

Fig. 4. Representative X-ray diffraction patterns of the phases, determined as described in the text and shown in Fig. 3. (a), (b), and (c) are of DOPE in 1 M trehalose, (d) is of DOPE in excess water. The high-angle line (double arrow) is at 4.1 Å showing crystalline hydrocarbon chains. The lines more central to these are of a 4.87 Å line from added teflon used as an internal standard. (a) $T = -5°C$: the population of low-angle reflections all fit in hexagonal two-dimensional symmetry, providing the existence of a single hexagonal phase. (b) $T = -10°C$: the low-angle reflections make up two independent sets of spacings; one indexes as a one-dimensional structure, and one as a two-dimensional hexagonal structure. This proves the coexistence of a lamellar and hexagonal phase. (c) $T = -12°C$: single lamellar phase with crystalline hydrocarbon chains. (d) $T = -5°C$: single lamellar (L_α) phase with disordered hydrocarbon chains. Comparison of (a) and (d) indicate directly the effect of trehalose in eliminating the L_α phase of DOPE.

$+12°C$, and H_{II} above $+12°C$. However, while attempting to identify the transition temperatures precisely it became clear that the temperature range of coexistence of the L_α and H_{II} phases depended on the time the sample spent in the -10 to $+12°C$ range. In fact if, over the period of 2–3 days, the temperature is slowly raised, the coexistence of L_α and H_{II} phases can be seen as high as at least $+15°C$. This result confirms similar observations by Tate and Gruner [12]. In addition, Shyamsunder et al. [13] have shown that DOPE can be trapped into a cubic phase by many temperature cycles in the -5 to $+15°C$ range. Importantly, this indicates that the single L_α phase is metastable and that hysteresis and equilibrium between the L_α and H_{II} phases are complicated, and likely involve a third isotropic phase as seen with NMR. We have not, therefore, studied this transition further. In the presence of trehalose no L_α phase is observed. Coexistence of L_β and H_{II} phases, with decreasing amounts of the former, occurs as the temperature is raised from -11 to $-8°C$. A pure H_{II} phase exists above $-8°C$. Thus, in the presence of trehalose, there is no intermediate L_α phase in the transition.

Discussion

Two new observations emerge from these studies. The first concerns the effect of trehalose on the structures formed by DOPE. The second concerns the metastability of the L_α phase of DOPE. Both the calorimetric and FTIR results suggest that DOPE, hydrated in a 1 M trehalose solution exhibits a large gel to liquid-crystalline transition similar to that shown in pure water. The absence of a second transition, as shown in Figs. 1 and 2, was originally interpreted by us to indicate the absence of a lamellar to H_{II} transition in the presence of the trehalose. The X-ray diffraction data presented here clearly demonstrate, rather, that trehalose causes the formation of the H_{II} phase directly from the lamellar gel phase. The enthalpy of an $L_{\beta'}$ to H_{II} transition may not be very different from an $L_{\beta'}$ to L_α transition. Since we were unable to obtain calorimetric enthalpies for our DOPE samples, we were unable to resolve any such difference.

Correlations between the transitions seen for DOPE in water in Figs. 1 and 2 with those of Fig. 3 show that the former detect the phase boundaries but do not determine what the transitions are. For example, the $+8°C$ transition represents the L_α-to-H_{II} transition if the temperature change is rapid enough and from a region well outside the -8 to $+12°C$ range [12], but represents the ($L_\alpha + H_{II}$)-to-H_{II} transition if the sample spent some time within this range (this study). Again, transitions are observed around $-8°C$; they represent the $L_{\beta'}$-to-L_α transition in water, but, unexpectedly, the $L_{\beta'}$-to-H_{II} transition in trehalose solution as described above. These observations emphasize the caution that must be exercised in translating observed transitions into structural changes. Similarly, it would appear to be an even more difficult matter to interpret shifts in transition temperature to shifts in phase boundaries and therefore to specific changes in structure. Such shifts have been seen in a number of systems similar to the present one but the interpretation in structural terms is not clear (see, for example, Ref. 14).

Somewhat similar results, using various sugars and sugar alcohols and dielaidoylphosphatidylethanolamine (DEPE) appeared while this report was in preparation [15]. However, in the case of DEPE in the presence of these solutes, the bilayer to hexagonal transition did not disappear, but occurred at a lower temperature relative to the transition in water. These authors also assume an inverted hexagonal (H_{II}) phase, a reasonable assumption since they were still able to detect a bilayer to hexagonal transition both calorimetrically and spectroscopically [15], albeit at an altered temperature. The amount of destabilization of the bilayer depended on the category of sugar, thus it appears that both the kind of solute and the particular species of PE will affect the degree of destabilization.

The mechanism by which trehalose induces the bilayer to H_{II} transition is unknown. It cannot be ruled out that surface active contaminants, which are present in many sugars, may cause the observed effects. However, if this is the explanation, the same surface active contaminant must be present in many different kinds of sugars, different lots of the same sugar, sugars obtained from different sources, used in different laboratories, and sugars which have similar but not identical effects on differents kinds of phosphatidylethanolamines [15].

The dramatic removal of the L_α phase is unlikely to be caused by osmotic dehydration of the phospholipid [16]. Previous studies have suggested that glucose and sucrose appear to be excluded from a fraction of the water between phosphatidylcholine bilayers; they are nevertheless equilibrated with the aqueous phase in hydrated egg phosphatidylcholine. We expect the same considerations to apply to phosphatidylethanolamines hydrated in sugar solutions. Thus, the exclusion of sugars from part of the water between lamellae cannot create an osmotic force to bring lamellae together [16]. In addition, we have shown that DOPE in poly(ethylene glycol) solution of the same wt% as 1 M trehalose does not eliminate the L_α phase (unpublished). The poly(ethylene glycol) molecule, being too large to enter the aqueous compartment of any DOPE phase, acts osmotically to shrink that phase uniformly. So we conclude that the dramatic removal of the L_α in DOPE is not caused by osmotic dehydration of the lipid but may be related to a possible local dehydration of polar groups, depending on the disposition of the sugar in the aqueous space and its possible exclusion from the water around the polar group.

Acknowledgements

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