Trehalose-Induced Destabilization of Interdigitated Gel Phase in Dihexadecylphosphatidylcholine

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ABSTRACT Trehalose is believed to have the ability to protect some organisms against low temperatures. To clarify the cryoprotective mechanism of trehalose, the structure and the phase behavior of fully hydrated dihexadecylphosphatidylcholine (DHPC) membranes in the presence of various concentrations of trehalose were studied by means of differential scanning calorimetry (DSC), static x-ray diffraction, and simultaneous x-ray diffraction and DSC measurements. The temperature of the interdigitated gel (L_{β}(i))-to-ripple ($P_{\beta'}$) phase transition of DHPC decreases with a rise in trehalose concentration up to ~1.0 M. Above a trehalose concentration of ~1.0 M, no L_{β}(i) phase is observed. In this connection, the electron density profile calculated from the lamellar diffraction data in the presence of 1.6 M trehalose indicates that DHPC forms noninterdigitated bilayers below the $P_{\beta'}$ phase. It was concluded that trehalose destabilizes the L_{β}(i) phase of DHPC bilayers. This suggests that trehalose reduces the area at the interface between the lipid and water. The relation between this effect of trehalose and a low temperature tolerance was discussed from the viewpoint of cold-induced denaturation of proteins.

INTRODUCTION

It is well known that some sugars, such as trehalose or sucrose, protect some organisms not only from various stresses due to dryness (Crowe et al., 1984, 1987), but also from damage due to low temperatures. Storey et al. (1981) have reported that the concentration of trehalose in the metabolites of gall fly larvas acclimated to low temperatures is higher than that of normal gall fly larvas.

Two different hypotheses for explaining the role of sugars in anhydrobiosis, i.e., the ability to survive against dehydration and rehaydration, have been proposed, on the basis of experimental results in vivo and in vitro. One is the water replacement hypothesis (Crowe et al., 1987; Crowe and Crowe, 1988). The main point of this hypothesis is that in a dehydration state, sugar molecules act as substitutes for water molecules by forming hydrogen bonds with the surfaces of proteins or cell membranes. The other hypothesis is based on the fact that concentrated sugar solutions can convert to a glassy state. In the capsule of the glassy sugar-water mixtures, proteins and cell membranes might be expected to be kept in their native states (Franks et al., 1991). This is called the vitrification hypothesis.

As compared with the intensive studies on the role of sugars in anhydrobiosis, the studies on the molecular mechanism of the protective function against damage due to low temperatures are not advanced at present. However, several speculations have been proposed. At least the presence of sugars restrains the formation of ice, which causes mechanical damage by increasing the volume. However, there are some cases in which organisms and cells suffer damage at low temperatures without the formation of ice. Quinn (1985) has considered the general function of cryoprotective agents at low temperatures, based on a lipid lateral phase separation, the formation of a nonbilayer structure in cell membranes, and the effects of cryoprotective agents on the phase behavior of phospholipid membranes. In this connection, Koynova et al. (1989) have recently proposed a hypothesis that explains the changes in phase transition temperatures of fully hydrated phospholipid membranes by the presence of sugars (trehalose or sucrose) (Chowdhry et al., 1984; Bryszewska and Epand, 1988; Koynova et al., 1989; Sanderson et al., 1991; Tsvetkov et al., 1989; Tsonev et al., 1994; Koynova and Caffrey, 1994). According to their hypothesis, the changes in the phase transition temperatures are considered a manifestation of the Hofmeister effect (Collins and Washabaugh, 1985), in which the sugars act as kosmotropic regents, i.e., the sugars stabilize the structure of water. Kosmotropic reagents raise the interfacial free energy between the lipid headgroup and the aqueous phases. This means that the presence of the sugars reduces the area of the interface between lipid and water. Consequently, the sugars stabilize the phases with a smaller interfacial area (e.g., normal gel ($L_{\beta'}$) or inverted hexagonal phases) and destabilize the phases with a larger interfacial area (e.g., liquid crystalline (L_{α}) phase).

Nishiwaki et al. (1990) have measured $^{31}P\{^{1}H\}$ nuclear Overhauser enhancement for unilamellar dipalmitoylphosphatidylcholine (DPPC) vesicles in trehalose solution. They have interpreted the result as indicating the increased packing density of the DPPC headgroup, induced by the presence of trehalose. On the other hand, an x-ray diffraction study has indicated that the addition of trehalose hardly affects the chain packing of multilamellar DPPC vesicles (Tsvetkov et al., 1989). Furthermore, there is a report which showed that a phospholipid monolayer on an aqueous phase is expanded laterally when trehalose is added to the aqueous

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subphase (Johnston et al., 1984). For this result, Arnett et al. (1986) have argued that the expansion of the lipid monolayers is due to not trehalose, but to impurities contained in the trehalose reagent used in the above experiments. In any case, it is unclear at present whether the interfacial area of lipid membranes is decreased by the presence of sugars.

The aim of this study is to examine the above problem. For this purpose, we studied the effect of trehalose on the phase behavior of multilamellar dihexadecylphosphatidylcholine (DHPC) vesicles. The previous experiments did not provide a clear conclusion, probably because the systems used in these experiments exhibited only a small change in the packing density of the lipid when trehalose is added. DHPC was chosen because it forms an interdigitated gel ($L_{\beta}(i)$) phase below a ripple ($P_{\beta'}$) phase (Ruocco et al., 1985; Kim et al., 1987; Laggner et al., 1987). As shown in Fig. 1, in the $L_{\beta}(i)$ phase, the surface area at the interface per lipid molecule is about twice that at a normal gel ($L_{\beta'}$) phase bilayer. It is therefore expected that trehalose should considerably affect the $L_{\beta}(i)$ phase of DHPC membranes if the hypothesis proposed by Koynova et al. (1989) is right.

Differential scanning calorimetry (DSC), static x-ray diffraction, and simultaneous x-ray diffraction and DSC measurements were performed to study the structural and thermal properties of DHPC in the presence of trehalose. We found that trehalose destabilizes the $L_{\beta}(i)$ phase of DHPC bilayers. On the basis of this result, we consider a speculation proposed by Franks (1985), according to which some sugars prevent proteins from undergoing cold-induced denaturation. Furthermore, from the viewpoint of the Hofmeister effect, we discuss the formation of the $L_{\beta}(i)$ phase of diacylphosphatidylcholines, induced by the addition of some small molecules.

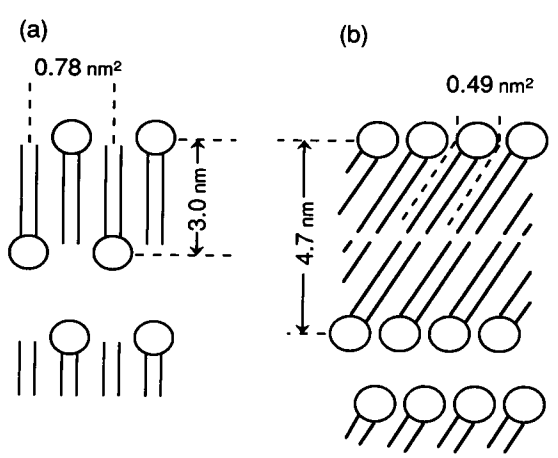

FIGURE 1 Schematic representation for the normal gel phase of DPPC and the $L_{\beta}(i)$ phase of DHPC. Circles and lines show the headgroups and the hydrocarbon chains, respectively. The distances and the area are according to the literature data (McIntosh et al., 1983; Nagle and Wiener, 1988; Laggner et al., 1987).

MATERIALS AND METHODS

Sample preparation

Powder of DHPC was obtained from Fluka (Buchs, Switzerland). The lipid gave a single spot on a silica gel thin-layer plate developed with $CHCl_3$/CH_3OH/H_2O (65:35:4, by volume), and it was therefore used without further purification. Trehalose was obtained from Sigma (St. Louis, MO). Arnett et al. (1986) have pointed out contamination of some surfactants in trehalose supplied by chemical companies. We purified the trehalose, using activated charcoal and recrystallization. After this procedure, we checked the purity of the trehalose with infrared spectroscopy. The water used in this study was prepared with a Mill-Q water purification system (Millipore Corp., MA).

A chloroform solution of DHPC in a small test tube was lyophilized under a stream of dry nitrogen, and the sample was kept under reduced pressure overnight. The lyophilized DHPC was dispersed into pure water or trehalose solutions with shaking at 60–70°C. The volumes of added pure water and trehalose solutions were 6 μl/4 mg DHPC for simultaneous x-ray diffraction and DSC measurements. In DSC measurements, 5 mg of DHPC was dispersed into 95 μl of water or trehalose solutions. For simultaneous x-ray diffraction and DSC measurements, the hydrated samples (~10 μl) were contained in a specially prepared aluminum cell, and the cells were covered with aluminum foil (Hatta et al., 1995; Takahashi et al., 1995). For static x-ray diffraction measurements, the samples were transferred to a 1.5-mm-diameter, fine-wall quartz capillary (Hilgenberg, Malsfeld, Germany).

Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were carried out with a DSC10/SSC580 system (Seiko I&E, Tokyo, Japan) at a scan rate of 1.0°C/min. Data acquisition and analyses were performed on a computer with software provided by Seiko I&E. The melting transition of high-purity gallium (99.9999%) was used to calibrate the scales of both the temperature and DSC thermogram. In this study, the transition temperatures of the lipid were simply defined by the position of the peak maximum. Before measurements, the samples were kept at 15°C for at least 30 min. Almost all of the samples were measured in the temperature range from 15°C to 60°C; however, the data for temperatures below 20°C are not shown in Fig. 3. No detectable peak was observed in the temperature range.

Simultaneous x-ray diffraction and differential scanning calorimetry

Simultaneous x-ray diffraction and DSC measurements were performed at station 15A at the Photon Factory in the National Laboratory for High-Energy Physics (KEK) at Tsukuba in Japan (Amemiya et al., 1983). In this station, an x-ray beam was focused and monochromatized with a bending fused silica mirror and a Ge crystal monochromator, respectively. The size of the incident x-rays was ~2 mm × 1 mm at the sample position. The wavelength of the x-rays was 0.1506 nm. X-ray diffraction patterns were recorded by using a position-sensitive proportional counter with 512 channels. The sample-to-detector distance was ~1.2 m. Spatial calibration was made by using the (001) diffraction from an anhydrous cholesterol powder sample. Data were stored in a SUN S-4/LX workstation. By using a differential scanning calorimeter (FP84; Mettler Instrument Corp., Hightstown, NJ) that had originally been developed for use in optical microscopy, we recorded not only time-resolved x-ray diffraction data, but thermal data simultaneously. The detailed setup of the apparatus has been reported elsewhere (Hatta et al., 1995; Takahashi et al., 1995). All experiments were performed with a heating scan at a rate of 1.0°C/min. In one scan, 94 consecutive diffraction patterns were recorded. The exposure time for each pattern was 20 s. Hence the total exposure time was ~33 min. In experiments using x-ray beams with high intensity, it is necessary to take into consideration the effects of radiation damage. To check the radiation damage, we observed the sequential diffraction profiles for 1 min of

continuous exposure for the pure DHPC at 45°C. Even after 40 min of exposure, the diffraction profile was almost unchanged. This result implies that the contribution of the radiation damage is not serious in this study, because no sample was exposed to the x-ray beam for more than 33 min.

Static x-ray diffraction

Static x-ray diffraction measurements were carried out by using a Ni-filtered CuKα radiation source (RU200BEH; Rigaku, Tokyo, Japan) and a two-dimensional area detector (imaging plate; Fuji Photo Film Co., Tokyo, Japan). An x-ray beam was focused by a double-mirror optical system. The capillary containing the sample was fixed to a hollow brass holder. Temperatures of the sample were controlled within 0.1°C by circulating water from a temperature-controlled water bath (B. Braun, Melsungen, Germany) to the sample mount. The data sampling of the imaging plates was performed with a BAS2000 data reading system (Fuji Photo Film Co.). Diffraction patterns were averaged by azimuthally integrating two-dimensional patterns (Takahashi et al., 1991) and were corrected by subtracting the background scattering obtained from the measurements of an empty capillary. The diffraction spacings were calibrated by using the (001) diffraction spacing of anhydrous cholesterol. The integrated intensities of the lamellar diffraction $I(h)$, where h is the diffraction order, were obtained by fitting the observed peak profile with the Lorentzian lineshape function. The magnitude of the structure factor $F(h)$ was set equal to $[h^2I(h)]^{1/2}$ (Blaurock and Worthington, 1966).

Phase determination

To estimate the membrane thickness of DHPC bilayers at 20°C in pure water and 1.6 M trehalose solution, the electron density profiles were calculated from the lamellar diffraction data. The information on the phase angles for each diffraction is needed to calculate the profiles. We determined it as follows. Judging from the chemical structure, it can be assumed that the structure of lipid membranes is centrosymmetrical. Therefore it is necessary to know only the sign (+ or −) of the phase angles. For the fully hydrated DHPC bilayers in water, we used the phase set (−, −, +, −, −), which had already been assigned by Kim et al. (1987). The phase set is identical with that of the interdigitated structure of DPPC induced by the addition of some small molecules (McIntosh et al., 1983; McDaniel et al., 1983). The phase set has been determined from an analysis for diffraction data obtained from the samples with various hydration levels by the use of the Shannon sampling theorem (Sayre, 1952). This method (so-called swelling method) has been widely used to determine the phase angle set for various membrane systems (Moody, 1963; Worthington and Blaurock, 1969; Torbet and Wilkins, 1976; Franks, 1976; McIntosh et al., 1983; McDaniel et al., 1983; MacNaughtan et al., 1985). We used the same method to determine the phase set for the DHPC in 1.6 M trehalose solution. The lamellar spacings were changed in the range from 6.80 nm to 5.99 nm by varying the amount of added 1.6 M trehalose solution to the lipid in the range from 26 wt% to 63 wt%. The observed intensity data sets were normalized using the following equation (Worthington and Blaurock, 1969; Worthington, 1969):

$$\sum_{h=1}^{h=h_{max}} I(h) \propto \frac{d}{d_{min}} \qquad (1)$$

where h_{max} is the maximum order observed, d is the observed lamellar spacing, and d_{min} represents the minimum lamellar spacing (5.99 nm) in the swelling experiment. The magnitudes of the normalized structure factors ($|F(h)|$) were plotted against reciprocal space (S) (= 2 sin θ/λ; 2θ = scattering angle, λ = wavelength of x-rays) (figure not shown). In the figure, so as to trace the data points, we drew by eye a curve with three nodes in the range $0.1 < S < 0.9$ (nm^{-1}). Thus there are three regions, I ($\sim 0.1 < S < 0.33$ nm^{-1}), II ($0.33 < S < 0.5$ nm^{-1}), and III ($0.5 < S < 0.9$ nm^{-1}). In each region, the phase angles have the same sign. Hence there are $2^3 = 8$ possible combinations. For all possible combinations, continuous transforms were calculated by using the Shannon sampling theorem:

$$F(S) = \sum_{h=-h_{max}}^{h=h_{max}} F(h) \frac{\sin[\pi(dS - h)]}{\pi(dS - h)} \qquad (2)$$

The calculations were made for the two data sets, with lamellar spacings of d = 6.80 nm and d = 5.99 nm. For the correct phases, the continuous transforms calculated for each data set should be the same. The calculation also requires the value of $F(0)$, which cannot be obtained experimentally. At first, we assumed that $F(0) = 0$, because the term of $F(0)$ contributes mainly near the zero of reciprocal space (Franks, 1976). As a result, the possibility of phase combination for each region was limited to four cases ((−, +, −), (+, −, +), (−, +, +), and (+, −, −)). Next, by taking $F(0)$ as a parameter, $F(0)$ was determined by fitting all of the experimental data with the continuous curve calculated from Eq. 2. On the other hand, $F(0)$ can also be calculated from chemical compositions, mass densities, and the unit cell volume (MacNaughtan et al., 1985; Nagle and Wiener, 1989). In this study, the unit cell could not be determined; however, only the sign of $F(0)$ can be estimated to be minus from the mass densities of 1.6 M trehalose solution (~1.12 g/ml) and DHPC (~1.0 g/ml). From both of the estimations of the sign of $F(0)$, we concluded that the correct phase is either (−, +, −) or (+, −, −) for the three regions I, II, III. Comparing them, the former combination was chosen on the basis of the assumption that the electron density level of the headgroup region of the bilayer should be higher that of the hydrocarbon core. Fig. 2 represents the plot of the structure factors and the continuous Fourier transform calculated with Eq. 2. From the above procedure, we determined that the phase combination for each lamellar diffraction is (−, −, +, −, −) for the fully hydrated DHPC bilayer in the 1.6 M trehalose solution.

RESULTS

DSC

Fully hydrated DHPC multibilayer vesicles exhibit three different phases separated by two phase transitions: pretransition at 35.4°C and main transition at 43.5°C, in the temperature range from 20°C to 60°C (Ruocco et al., 1985; Laggner et al., 1987; Kim et al., 1987; Cunningham et al., 1995). Differing from the case of DPPC bilayers, the pretransition of DHPC bilayers was a transition from the L_β(i) to the ripple ($P_{\beta'}$) phases.

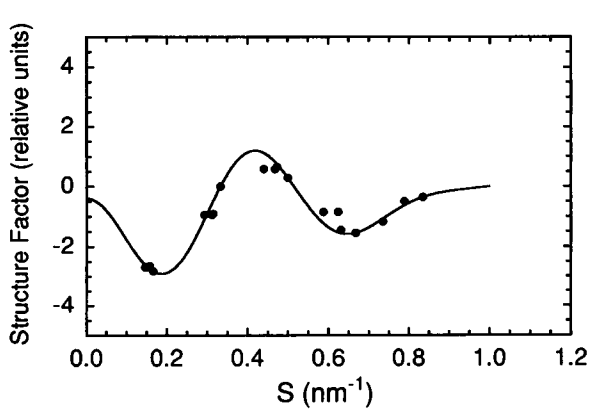

FIGURE 2 Plot of the normalized structure factors of DHPC in 1.6 M trehalose solution at 20°C as a function of the reciprocal space (S). The line corresponds to the continuous Fourier transform calculated from the data of d = 5.99 nm, by using the Shannon sampling theorem.

The effect of trehalose on the phase transitions of DHPC bilayers is presented in Fig. 3. This figure shows that an increase in the trehalose concentration leads to a linear rise in the main transition temperature and a linear fall in the pretransition temperature. This effect is characterized quantitatively by slopes of −9.2°C/mol and 1.2°C/mol for the pretransition and main transition, respectively. The former value is almost the same as that of DPPC in the presence of trehalose (0.8°C/mol) (Tsvetkov et al., 1989). Up to the trehalose concentration of 0.8 M, the peak of the pretransition appears in the DSC thermograms. Above 1.0 M, however, the peak of the pretransition disappears from the thermogram.

For the DSC curves for a trehalose concentration above 0.8 M, an additional small endothermic peak is observed at ~44°C, i.e., ~1°C lower than the main transition. The origin of this small peak is unclear at present. Recently, Jørgensen (1995) has reported the existence of a similar small transition peak that appears ~1°C lower than the main transition in long-chain phosphatidylcholines.

Simultaneous x-ray diffraction and DSC

Fig. 4 displays three-dimensional plots of x-ray scattering intensity versus reciprocal space (S) as a function of temperature for a pure DHPC bilayer and several DHPC-trehalose systems obtained by simultaneous x-ray diffraction and DSC measurements. To compare the x-ray diffraction data with the DSC data obtained simultaneously, we arranged the two-dimensional projection of the x-ray diffraction data and the DSC thermograms side by side in Fig. 5. In the DSC thermograms of this figure, in contrast to the usual way, the vertical axis is temperature and the horizontal axis is heat flow. The DSC thermograms obtained simultaneously are essentially identical to these obtained by the above normal DSC measurements (Fig. 3). For the DHPC bilayer in the presence of 1.2 M trehalose, however, the small endothermic peak at ~44°C was not clearly observed (Fig. 5 *d*), as compared with the data obtained with the normal DSC. This might be due to the difference in the sensitivity and resolution of temperature between the DSC apparatuses used in the two measurements. In the thermogram of DHPC in 1.6 M trehalose solution, a small peak was clearly observed (Fig. 5 *e*). It can be seen from Figs. 4 and 5 (especially Fig. 5) that structural changes are almost correlated with the thermal event. Exceptions are structural changes in DHPC in the presence of 1.2 M and 1.6 M trehalose. The x-ray diffraction patterns change at ~24°C and ~30°C for 1.2 M and 1.6 M trehalose, respectively, despite the fact that no distinct peak was observed in the present DSC thermograms (see Discussion).

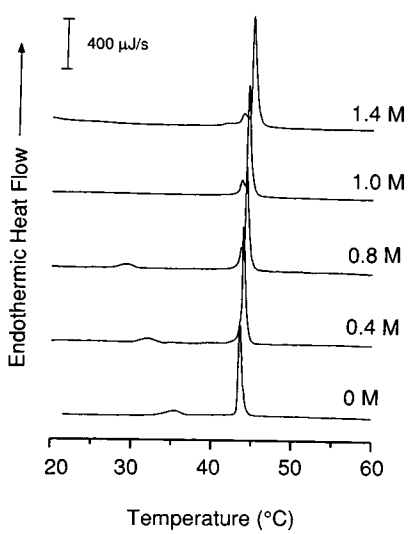

FIGURE 3 Typical DSC thermograms for pure DHPC and DHPC in the presence of trehalose. The heating scan rate was 1.0°C/min. The weight of the lipids was 0.75 mg. The trehalose concentrations are indicated on the right side of each thermogram.

The spacings estimated from the lowest diffraction peak at 20°C are plotted in Fig. 6 as a function of the trehalose concentration. These spacings are assigned to lamellar spacings, except for the datum of 1.0 M trehalose. At present it is not clear whether DHPC in the presence of 1.0 M trehalose at 20°C is in the $L_{\beta'}$ or the $P_{\beta'}$ phases. This problem will be discussed in the Discussion. The error in the data for the case of 1.0 M trehalose is greater than that of other data. This is due to the broadness of the diffraction peak around 20°C. The longer lamellar spacings in the presence above ~1.0 M trehalose suggest that the DHPC molecules do not form the interdigitated structure at 20°C. The lamellar spacing is composed of the sum of the thickness of membranes and the aqueous layers. Therefore, we cannot conclude from the data of the lamellar spacings alone whether the DHPC bilayers assume an interdigitated structure, or the water layer between the adjoining bilayers becomes wider in the interdigitated structure. This problem will be solved by reconstructing an electron density profile from the intensity data of the lamellar diffractions in the next section.

Trehalose affects both structures of the $P_{\beta'}$ and L_α phases in the presence of 1.2 M trehalose. The effect on the $P_{\beta'}$ phase will be described elsewhere (Takahashi et al., in preparation). With regard to the effect on the L_α phase, it should be pointed out that Cunningham et al. (1995) have observed similar x-ray lamellar reflection profiles with a shoulder in the wider angle region for the L_α phase DHPC bilayers in the presence of small amounts of sterols. They have interpreted the shoulder of the diffraction peak as suggesting the formation of a ripple structure in the liquid crystalline phase. We shall, however, make no further inquiry into this problem, because we do not have sufficient data about this phenomenon.

Static x-ray diffraction and electron density profile

Static x-ray diffraction patterns for pure DHPC bilayers and DHPC bilayers in the presence of 1.6 M trehalose at 20°C

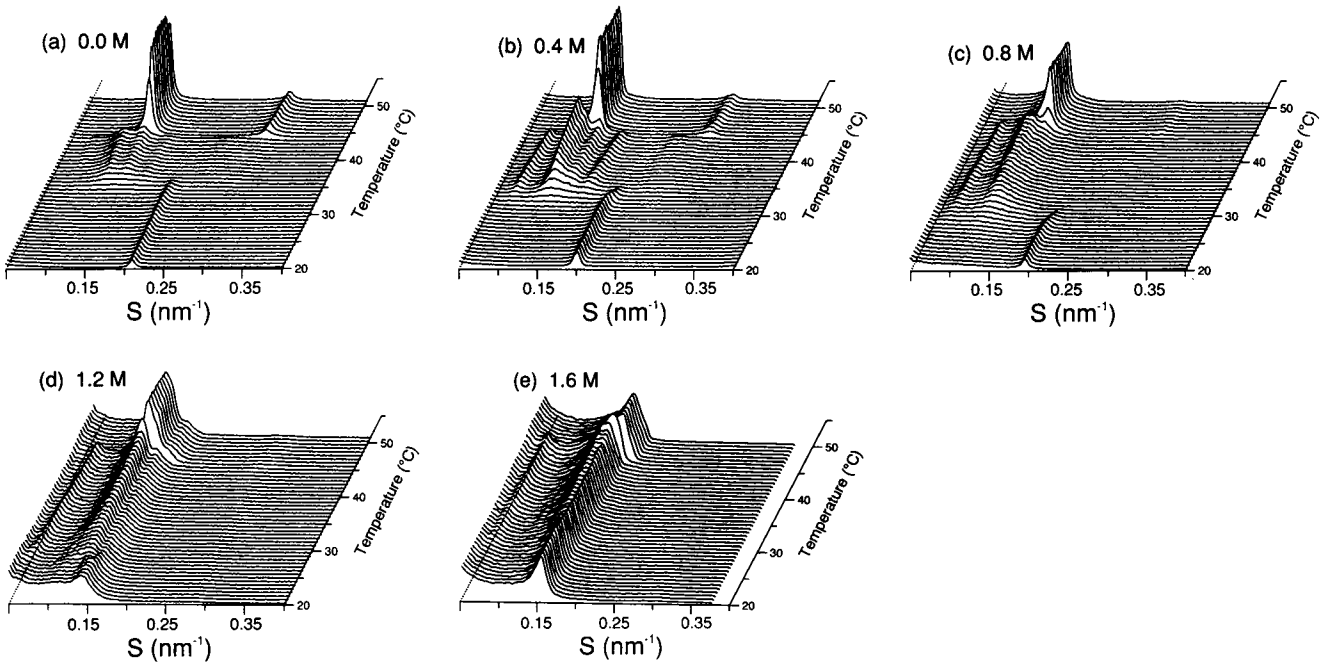

FIGURE 4 Three-dimensional plots of x-ray diffraction patterns as a function of constantly increasing temperature (1.0°C/min) for (*a*) pure DHPC and DHPC in the presence of (*b*) 0.4 M, (*c*) 0.8 M, (*d*) 1.2 M, and (*e*) 1.6 M trehalose. Every second diffraction pattern from the total set of 94 consecutive diffraction patterns is shown.

are shown in Fig. 7, *a* and *b*, respectively. The lamellar spacings are 4.79 nm and 6.80 nm for the pure DHPC and the DHPC with 1.6 M trehalose, respectively. These spacings are in good agreement with those determined by the above simultaneous x-ray diffraction and DSC measurements. As shown in Fig. 7, the lamellar reflections were detected up to the fifth order for both samples. The disappearance of the fourth-order lamellar reflection for pure DHPC bilayers might be due to its weak intensity.

From these x-ray diffraction data, we calculated the electron density profiles of pure DHPC and the DHPC in the presence of 1.6 M trehalose. The electron density profile of the DHPC bilayers at 20°C is presented in the upper right of Fig. 7 *a*. The highest peak of the profile shows the position of the polar headgroups of the DHPC bilayers. Accordingly, the profile indicates that the thickness of the bilayer is short (2.98 nm). This is a common characteristic of lipid bilayers with interdigitated hydrocarbon chains (McIntosh et al., 1983; Simon and McIntosh, 1984; Ruocco et al., 1985; Kim et al., 1987; Laggner et al., 1987; Adachi et al., 1995). In contrast, the election density profile shown in the upper right of Fig. 7 *b* indicates that the DHPC in the presence of 1.6 M trehalose has relatively thicker bilayers (4.44 nm). This profile is almost identical with that of the $L_{\beta'}$ phase of DPPC bilayers with no interdigitated structure (Luzzati, 1967; Levine and Wilkins, 1971; Torbet and Wilkins, 1976; Inoko and Mitsui, 1978; McIntosh, 1980; McIntosh and Simon, 1986). From this result, it can be concluded that the increase in the lamellar spacings above ~1.0 M trehalose (Fig. 6) is due to the increase in the thickness of the bilayers, i.e., in the presence of trehalose above 1.0 M, the DHPC forms the normal gel phase bilayer structure below the $P_{\beta'}$ phase.

DISCUSSION

In this study the effect of trehalose on the phase behavior of DHPC membrane was studied by DSC and x-ray diffraction in the trehalose concentration range from 0 to 1.6 M. The results are schematically summarized in Fig. 8.

In the figure, dotted lines indicate the boundaries that could not be proved clearly. The distinct boundary between the L_β(i) and $P_{\beta'}$ phases in particular could not be determined. With respect to the boundary between the $L_{\beta'}$ and $P_{\beta'}$ phases, we plotted the temperatures at which a diffraction peak with a long spacing (> ~12 nm) appears in the diffractograms. The diffraction peak might be related to the ripple repeat distance. As seen in Figs. 4 *a* and 5 *a*, however, for pure DHPC bilayers, no distinct peak with a long spacing (> ~12 nm) appears in the x-ray diffraction profiles in the $P_{\beta'}$ phase. It is therefore not certain that the presence of the peak with a long spacing always indicates the appearance of a ripple phase. In addition, it is likely that the intensity of the diffraction is too weak to give rise to a detectable peak. This might be connected with the fact that the DHPC bilayer in 1.0 M trehalose solution at 20°C exhibits a longer lamellar spacing (Fig. 6). Because of the absence of a detectable peak with a long spacing (> ~12 nm) at 20°C, we assigned the $L_{\beta'}$ phase (Fig. 8). However,

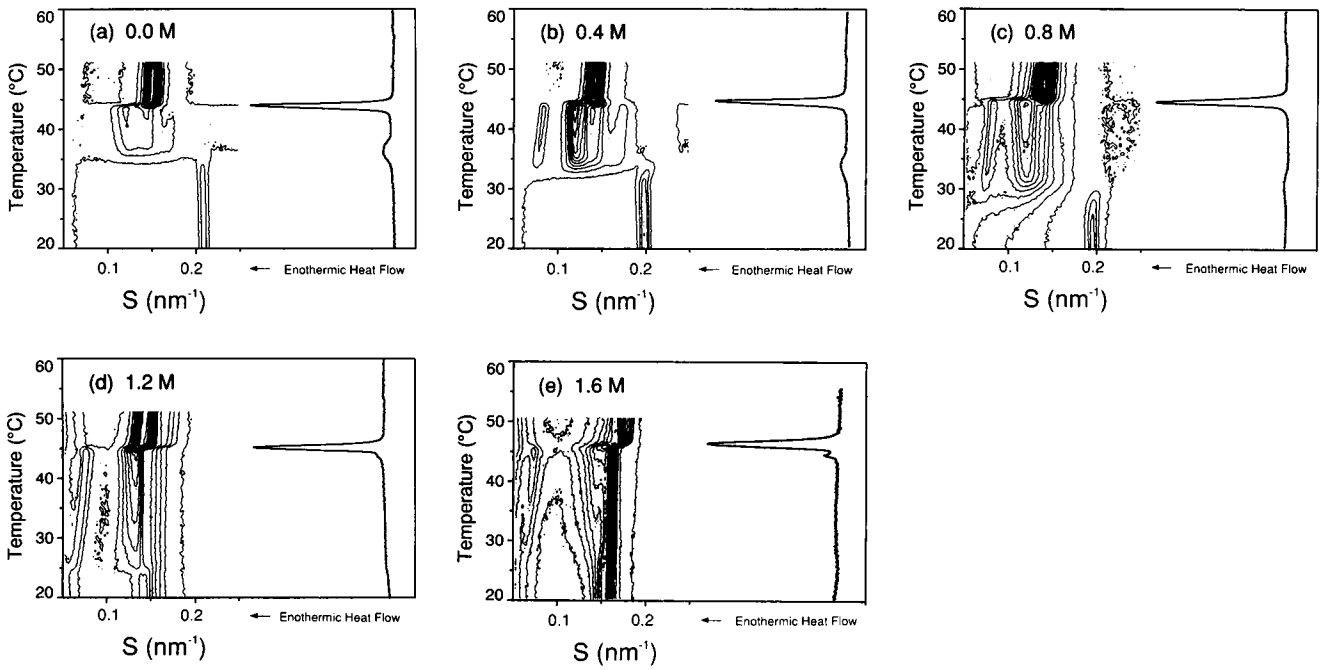

FIGURE 5 Comparison between DSC data and x-ray diffraction data obtained simultaneously for (*a*) pure DHPC and DHPC in the presence of (*b*) 0.4 M, (*c*) 0.8 M, (*d*) 1.2 M, and (*e*) 1.6 M trehalose. The x-ray diffraction data are shown in two-dimensional projection style on the left side of each figure. The DSC data are shown on the right side of each figure; the horizontal and vertical axes represent heat flow and temperature, respectively. In the DSC thermogram of *d*, there seems to be a break in the baseline around 25°C; however, the break was not observed in more sensitive normal DSC measurements (data not shown; see Fig. 3 for the case of 1.0 M and 1.4 M trehalose).

the DHPC-1.0 M trehalose system at 20°C might be in the $P_{\beta'}$ phase.

For DHPC bilayers in the presence of more than ~1.0 M trehalose, a peak associated with the pretransition ($L_{\beta'}$-to-$P_{\beta'}$) was not observed clearly in the DSC thermograms obtained from simultaneous measurements (see Fig. 5, *d* and *e*). On the other hand, a structural change was clearly detected by x-ray diffraction. This discrepancy between the DSC and x-ray diffraction results around 20–30°C is not due to the low sensitivity of the DSC apparatus used in the simultaneous measurements. Above 1.0 M trehalose, there was also no DSC peak around 20–30°C, even in the thermogram obtained with a standard DSC apparatus (see Fig. 3). Such a discrepancy has been reported for phosphatidylcholine (PC) bilayers containing more than 10 mol% cholesterol. In fact, no distinct peak due to the pretransition ($L_{\beta'}$-to-$P_{\beta'}$) appears in DSC thermograms in the case where more than ~4–10 mol% cholesterol was added to PC (Estep et al., 1978; Mabrey et al., 1978; Koynova et al., 1985; Vist and Davis, 1990; McMullen et al., 1993). Even at ~15 mol% cholesterol, however, the formation of the ripple structure has been detected by experiments with freeze-fracture (Copeland and McConnell, 1980; Hicks et al.,

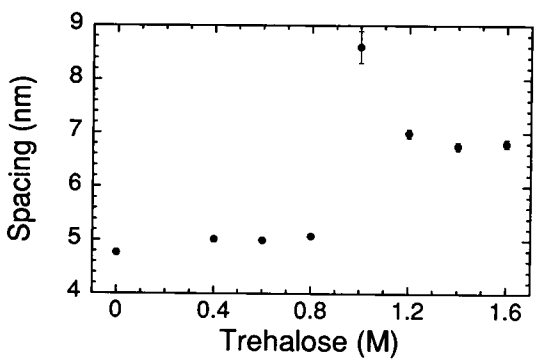

FIGURE 6 Spacings estimated from the lowest diffraction peak at 20°C for DHPC-trehalose systems as a function of the trehalose concentration.

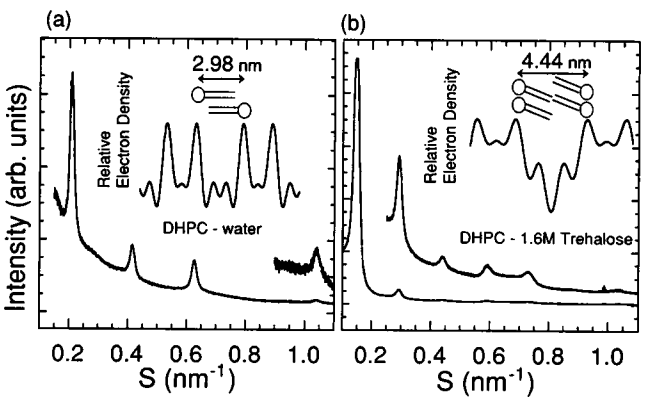

FIGURE 7 Static x-ray diffraction patterns recorded at 20°C and the relative electron density profiles for (*a*) pure DHPC and (*b*) DHPC in the presence of 1.6 M trehalose. For x-ray diffraction data in the wider angle region, the patterns are also shown in 10-fold expanded vertical scale.

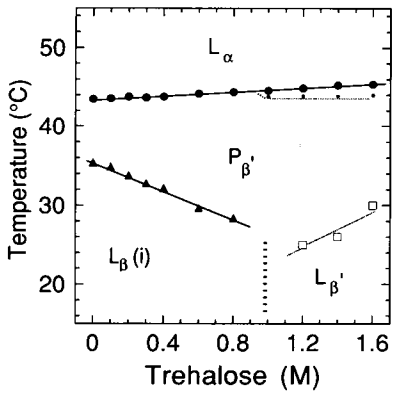

FIGURE 8 Trehalose concentration dependence of the phase behavior of DHPC. The points shown by closed symbols present the peak temperatures of DSC thermograms. The points shown by open symbols present the temperature at which a diffraction peak with a long spacing (> ~12 nm) appears in the x-ray diffractograms obtained from simultaneous measurements (see text).

1987) and neutron diffraction (Mortensen et al., 1988). A recent time-resolved x-ray diffraction study of dimyristoylphosphatidylcholine-cholesterol systems has suggested that the absence of a peak in the DSC thermograms for PC containing more than 10 mol% cholesterol is due to broadening of the thermal peak, i.e., there is two-phase coexistence (Matuoka et al., 1994). In the present case, however, we could not clearly conclude whether there is two-phase coexistence, owing to the low resolution of the x-ray diffraction patterns (Figs. 4 *d*, 4 *e*, 5 *d*, and 5 *e*) and a lack of knowledge about the structure of the $P_{\beta'}$ phase of DHPC bilayers. More detailed studies are required to solve this problem. It should be pointed out that the diffraction pattern of the $P_{\beta'}$ phase of DHPC bilayers is rather more similar to that of the metastable $P_{\beta'}$ phase of DPPC bilayers (Yao et al., 1991; Rappolt and Rapp, 1996) than to that of the normal $P_{\beta'}$ phase (compare Fig. 4 with figures 1 and 2 of Yao et al., 1991).

The important result in the present study is that trehalose destabilizes the L_β(i) phase of DHPC bilayers, i.e., the presence of trehalose at concentrations above 1.0 M induces normal $L_{\beta'}$ phase bilayer. This indicates that the surface area at the interface between the water layer and the lipid bilayer drastically decreases in the presence of trehalose. This ability of trehalose to reduce the interfacial area has been discussed, based on the effect of trehalose on the phase transition temperatures of phospholipid membranes (Koynova et al., 1989). The present result clearly shows the reduction of the interfacial area. This can be also interpreted by the fact that trehalose behaves as a kosmotropic regent, i.e., a water-structure maker (Collins and Washabaugh, 1985). It has been reported that both calcium ions (Kõiv and Kinnunen, 1992) and deuterium oxides (Ohki, 1991) decrease the pretransition temperature of DHPC bilayers, i.e., these molecules destabilize the L_β(i) phase of DHPC bilayers. These molecules are also classified into water-structure makers (Collins and Washabaugh, 1985).

We consider the relation of the result obtained in the present study with the role of trehalose in the tolerance by some organisms for low temperatures. It is known that proteins undergo cold-induced denaturation, i.e., proteins do not adopt a native state at subzero temperatures (see reviews by Privalov and Gill, 1988, and Franks and Hatley, 1991). When proteins undergo denaturation, their functional capabilities disappear. In the native state of proteins, the hydrophobic amino acid residues of proteins are located in an inside region, and only hydrophilic amino acid residues make contact with surrounding water molecules. In the denatured state, because of the break in the secondary and higher order structures, the hydrophobic residues of proteins are exposed to water, and the interfacial area in contact with water increases in comparison with the native state. If the origin of reducing interfacial areas of lipid bilayers induced by trehalose is due to the stabilization of water structure (indirect effect), the role of trehalose might also be applied for all amphipathic molecules, including proteins. According to this assumption, it may be concluded that by reducing the interfacial area, trehalose stabilizes the native state of proteins, even at subzero temperatures, i.e., trehalose prevents proteins from undergoing cold-induced denaturation. This might be correlated with the resistance to low temperatures created by the production of trehalose in some organisms. It has been pointed out that the thermodynamic properties of cold-induced denaturation of proteins are similar to those of heat-induced denaturation (Franks, 1985; Privalov and Gill, 1988; Franks and Hatley, 1991). Sugars and sugar alcohols have been reported to stabilize the native state of proteins against heat-induced denaturation (Gekko and Morikawa, 1981; Arakawa and Timasheff, 1982). On the basis of these facts, Franks (1985) has already speculated that some sugars and sugar alcohols also stabilize the native state of proteins against cold-induced denaturation. The present result supports Franks' speculation in a sense.

The chaotropic reagents have properties that are the reverse of those of the kosmotorpoic reagents. From this point, we consider the formation of the L_β(i) phase of diacylphospholipids by the addition of small molecules. Actually, small molecules that induce the L_β(i) phase for diacylphospholipids, i.e., alcohols (Simon and McIntosh, 1984; Nambi et al., 1988), KSCN (Cunningham and Lis, 1986), choline (Ranck and Tocanne, 1982), acetone (Kinoshita et al., 1997), acetylcholine (Ranck and Tocanne, 1982), and glycerol (McDaniel et al., 1983; Swamy and Marsh, 1995), are all chaotropic reagents, in the sense that they lower the surface tension of the air-water interface. These facts suggest that the formation of the L_β(i) phase by the addition of small molecules may be explained in terms of the Hofmeister effect. The presence of chaotropic reagents (e.g., ethanol) causes the surface area per lipid to expand. It is expected at least that the expansion of the surface area acts as a trigger for the formation of the L_β(i) phase. A similar concept has already been discussed (Ohki, 1991; Crowe et al., 1993; Kinoshita and Yamazaki, 1996).

Glycerol stabilizes the native state of proteins, differing from the other chaotropic materials described above (Gekko and Timasheff, 1981). Gekko and Timasheff (1981) have proposed that the protein stabilization mechanism of glycerol must be due to the repulsive force between glycerol and nonpolar regions located on the protein surface, because glycerol is essentially as hydrophilic as water is, and that glycerol may penetrate the solvation sheath of protein. Similarly, it is also necessary to consider the direct penetration of small added molecules into the interfacial region of membranes for interdigitated structure formation. Recently, an x-ray diffraction study by Adachi et al. (1995) has strongly suggested such a penetration mechanism of ethanol in the interdigitated structure of diacylphosphatidylcholines.

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