Localization of trehalose in partially hydrated DOPC bilayers: insights into cryoprotective mechanisms

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Trehalose, a natural disaccharide with bioprotective properties, is widely recognized for its ability to preserve biological membranes during freezing and dehydration events. Despite debate over the molecular mechanisms by which this is achieved, and that different mechanisms imply quite different distributions of trehalose molecules with respect to the bilayer, there are no direct experimental data describing the location of trehalose within lipid bilayer membrane systems during dehydration. Here, we use neutron membrane diffraction to conclusively show that the trehalose distribution in a dioleoylphosphatidylcholine (DOPC) system follows a Gaussian profile centred in the water layer between bilayers. The absence of any preference for localizing near the lipid headgroups of the bilayers indicates that the bioprotective effects of trehalose at physiologically relevant concentrations are the result of non-specific mechanisms that do not rely on direct interactions with the lipid headgroups.

1. Introduction

Small solute molecules, in particular sugar molecules, are associated with cryo- and anhydro-protection of membranes in various organisms. During drying or slow cooling at moderate sugar concentrations, in which kinetic arrest of the system owing to the formation of a glass by sugars [1–3] is not important, the primary mode of protection is the avoidance of lipid phase transitions that can interfere with the intrinsic transport properties of the membrane [4,5].

Two molecular scale theories have been proposed to explain how sugars are able to protect cell membranes from these deleterious phase transitions. The first was the water replacement hypothesis (WRH) [6], which was based on specific interactions occurring between lipid headgroups and the protecting sugar molecule, with the sugars replacing water molecules around the lipid headgroup at low hydrations [4,7–9]. This hypothesis originally ascribed a specific and special effect to trehalose, although this was broadened to other disaccharides in later work [9]. Evidence for this hypothesis was largely built on the effects of trehalose on the phase transition temperature of dry phospholipids [10], and supported by measurements of the effects of trehalose on the surface pressure isotherms of monolayers of lipids [11], changes of Fourier transform infrared spectra in the presence of sugars at low hydration [12] and molecular dynamics simulations of bilayer/trehalose systems [13,14]. An alternative theory is the hydration forces explanation (HFE), which invokes the mediation of the forces between bilayers by the sugars as membranes come close together, and the subsequent reduction in the induced lateral compression in the plane of the bilayer responsible for deleterious phase transitions [15–20]. This theory is supported by direct measurement of the forces between bilayers [21], solid-state nuclear magnetic resonance (NMR) [22], and more
Figure 1. Cartoon illustrating two potential scenarios for the location of sugars in a lipid membrane system. In (a), most of the solute is excluded from the bilayer surface; in (b), specific interactions between the sugar and lipid headgroups result in higher concentrations of sugar bound to the bilayer surface. (Online version in colour.)

recently a range of X-ray and neutron scattering studies [17–20,23,24]. Moreover, this explanation provides a quantitative prediction of the effects of sugars on the fluid–gel transition temperature as a function of hydration [19].

Recently, the two opposing views have been reconciled on the basis of a concentration-dependent behaviour, where at lower concentrations sugar is selectively partitioned into a finite number of sites, and at high concentrations these sites are saturated, and the sugar molecules behave as normal molecules in solution [25]. However, debate continues around the dominant mechanism of membrane protection during dehydration. This is due, in part, to the fact that the early work was largely carried out on dry systems, leading to conclusions that cannot be extended to other hydrations, but have pervaded the literature. In addition, almost all the experimental work to date has used indirect methods to infer the role of the sugars within the membrane/water system.

In order to finally resolve this issue, we turn to a direct method of determining the location of the sugars within a membrane system at physiologically relevant sugar and water concentrations. The WRH and HFE imply quite different concentration profiles of sugar around the bilayer/water interface. In the case of the WRH, owing to the proposed interaction, there should be an enhancement of the sugar concentration in the region of the headgroups, and in the case of the HFE, the inter-bilayer water should behave as a simple aqueous solution of trehalose. This difference is schematically shown in figure 1.

Neutron and X-ray membrane diffraction of stacked aligned lipid bilayer phases has proved to be a powerful tool to understand bilayer structure [26–33] and probe the interactions between introduced molecules and bilayers [34–38]. This is accomplished by calculating scattering difference profiles that show the contribution to the unit cell profile of the introduced molecule. This method benefits greatly from neutron scattering combined with isotopic substitution, which allows precise determination of the scattering length density (SLD) contributions from molecules that have been selectively deuterated, and allows for the accurate phasing of the Fourier reconstruction.

We present here the results of neutron membrane diffraction measurements on highly aligned lipid bilayer stacks as a direct probe of the location of sugars within a bilayer system at intermediate hydration. By varying the SLD of the trehalose through isotopic substitution, the contribution of the sugar molecules can be differentiated from the SLD profile of the system and the location of the sugar molecules can be determined directly for the first time.

2. Material and methods

Dioleoylphosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids and used without further purification. Protonated trehalose was obtained from Sigma Aldrich. Deuterated trehalose was produced by the National Deuteration Facility, ANSTO. This was achieved by catalytic exchange reactions following a procedure of Koch and Stuart using a Raney nickel catalyst [39,40]. Isotopic purity, determined by mass spectrometry and solution NMR, was 67.8% of the 14 non-exchangeable hydrogens in the trehalose molecule. DOPC and trehalose were dissolved in chloroform and methanol, respectively. Samples were made with trehalose/DOPC ratios of 0.5 : 1. This concentration was chosen to mimic natural biological systems that use sugars as a natural defence against dehydration and freezing damage to cell membranes. Solutions were mixed together in the required quantities and sprayed onto a quartz slide with an airbrush. The samples were placed in a vacuum chamber for several hours to remove all traces of chloroform and methanol. Hydration of samples was accomplished by placing them in a humidity cell with saturated K2SO4 solutions (RH = 97%) for 12 h to hydrate the samples via the gas phase. The DOPC subsequently self-assembled into stacks of lipid bilayers. Samples and salt solutions were sealed in aluminium canisters with saturated NaBr solutions (RH = 57%) at least 12 h prior to measurement, so that measurement sample hydration and H2O/D2O exchange between the saturated salt solution and the sample reached equilibrium. Sample temperature was maintained at 25°C by circulating water through tubing spiralled around the Al canisters.

The solution layer shrinks and swells as a function of changing humidity. Sample thickness was determined gravimetrically, assuming uniform coverage across the substrate. Rocking curves were recorded for the first five diffraction orders for each sample at each of the three D2O/H2O ratios. Figure 2 shows a typical rocking scan for a first-order Bragg reflection. The full width at half maximum (FWHM) of 0.3 (2θ) indicates a low mosaic spread within the sample. The low mosaic spread of the samples indicates a sample which is effectively a one-dimensional crystal with the unit cell consisting of a lipid bilayer and a solution layer in equilibrium with the ambient humidity. Repeat spacings of the bilayers were 51.00 ± 0.02, 51.21 ± 0.02 and 50.77 ± 0.02 Å for protonated trehalose, protonated/deuterated trehalose mix and deuterated trehalose samples, respectively. There is a small variation of repeat spacing between samples of 0.2 Å.

Three percentages of trehalose dehydration were studied: 0% deuterated, 33.9% deuterated (a 50 : 50 mix of the protonated and deuterated trehalose) and 67.8% deuterated. Each sample was measured at three H2O/D2O ratios (8% D2O, 20% D2O and 50% D2O) by varying the H2O/D2O ratio of the saturated salt solution. The variation in the structure factors with the change of D2O in the water layer of the bilayer enables identification of the structure factor phases. See electronic supplementary material, figure S1.
3. Results and discussion

Figure 3 shows the measured SLDs for three samples with different percentages of trehalose deuteration. Figure 3 clearly shows that the presence of trehalose does not significantly alter the bilayer structure, but has a dramatic effect on the inter-bilayer aqueous layer. As hydrogenated trehalose is replaced by deuterated trehalose, the neutron contrast increases and the location of the trehalose molecules becomes clear. This is even more clearly illustrated by subtracting the protonated trehalose profile from the deuterated trehalose profile in real space. This results in a difference profile directly showing the trehalose contribution, as shown in figure 4b, where the representation has now been changed to have the centre of the water layer in the centre of the figure. The difference profile indicates a Gaussian distribution of the trehalose centred in the water layer, with the fit shown. The deviations from the fit at large distances from the centre of the water layer are due to Fourier truncation errors and experimental noise. It is clear that in the aqueous layer the data can be well described by a single Gaussian fit, implying that the bulk of the trehalose is concentrated towards the centre of the water layer.

Figure 4a shows for comparison the water distribution between the bilayers. Here, the distribution is broader, indicating that the water is distributed more evenly across the inter-bilayer space. Two Gaussian profiles are needed to fit this distribution, consistent with previous work [41]. These data clearly show that the trehalose is behaving like a free solute: there is no indication of a deviation away from this behaviour owing to preferential association of the trehalose with the lipid headgroups.

These results show unequivocally that the trehalose molecules are predominantly excluded from the membrane surface, and have their highest concentration in the centre of the water layer. This adds to the large body of experimental work which implies that, under physiologically relevant concentrations, sugars do not interact strongly with membrane lipids, and are largely excluded from the lipid surface. Evidence for this has come from solid-state NMR [22], surface forces measurements [21], differential scanning calorimetry [42] and small angle X-ray [19] and neutron [17,43] studies. A parallel body of work investigating the stabilization of proteins by solutes such as sugars [44–46] and glycerol [47,48] shows similar results: stabilization is due to non-specific effects, with the solutes being excluded from the surface of the proteins.
The fact that trehalose is largely excluded from the membrane demonstrates that its protective effects must be due to a mechanism that does not rely on direct interactions with the lipid headgroups. Thus, like any small uncharged solute of similar size, the presence of the trehalose reduces the osmotic forces acting on the system for a given environmental humidity (or osmotic pressure). In addition, their presence between opposing membranes prevents the close approach of the membranes. The ability of sugars to protect membranes under these conditions is therefore determined largely by their non-specific osmotic and volumetric effects, as proposed by the HFE.

Recently, a study proposed that the sugar–membrane interaction was a function of the sugar concentration [25], and this is perhaps the reason why different types of experiments have yielded conflicting results. This study found that, at low sugar concentrations (less than approx. 0.2 M), sugars bind to the lipid headgroups. At higher concentrations, sugars are gradually expelled from the lipid headgroup region to locate in the water layer. The results presented here are consistent with that hypothesis, as the sugar concentrations used in this study are 2.28 M. Previous work has shown that such concentrations are needed to provide significant protection. For example, in *Artemia salina* cysts, trehalose is present at concentrations of about 15% of the dry weight, corresponding to about 0.15 M in the hydrated state [49]. Concentrations will quickly rise above 0.2 M during dehydration. Similarly, it was found that a minimum internal trehalose concentration of 0.12 M was required to confer dehydration resistance on *Saccharomyces cerevisiae* cells [50]. Indeed, during dehydration or slow freezing, effective concentrations will always exceed approximately 0.2 M, so direct interactions are unlikely to be important except when there is virtually no water left in the system (when there is no water left, the membranes and sugars must necessarily be brought into contact).

Finally, these results disagree with a number of molecular dynamics simulations which show direct interactions between sugars and membranes. Sum et al. [51] simulated fully hydrated DOPC bilayers in the presence of low and intermediate trehalose and sucrose concentrations. Their simulations indicate that the sugar hydrogen bonds with the phosphate and ester groups of the lipids, resulting in concentrations of trehalose or sucrose molecules adjacent to the lipid headgroups higher than that in the bulk aqueous phase, and similar results have been found in other molecular dynamics simulations [13,14], although recent molecular dynamics studies have confirmed that the effects are very concentration dependent [52]. The results presented here suggest a possible reason for this discrepancy. Almost all the molecular dynamics simulations have been carried out at high hydration, and with a single bilayer surrounded by solution. However, in dehydrated systems, the presence of multiple membranes separated by small distances is the critical factor which is not taken into account in the simulations. Simulations of two or more bilayers with the solution layers in between may lead to very different results, and become directly comparable to the experiments reported here.

The results presented here show, for the first time, direct experimental evidence that trehalose, the quintessential membrane protectant found in nature, does not associate strongly with membranes, and that direct interactions are not the dominant effect of sugars at physiologically relevant concentrations. These results will have a significant impact on our understanding of how sugars protect membranes during dehydration and slow freezing.

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**References**


47. Gekko K, Timasheff SN. 1981 Mechanism of protein stabilization by glycerol—preferential hydration in glycerol-water mixtures. 


