Effects of Trehalose on the Water Structure in Yeast Cells as Studied by *in vivo* ^{1}H NMR Spectroscopy

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(Received July 24, 1995)

Trehalose-containing and -lacking yeast cells are prepared, and *in vivo* ^{1}H NMR spectra are measured in order to investigate effects of this sugar on water structure in the cells. The T_2 relaxation times for the intracellular water protons are measured as a function of water content in the yeast cells. The results indicate that the intracellular water is categorized into at least two groups: highly immobilized water ($T_2 \fallingdotseq 5$ ms), assigned to bound water, and intermediate water. One of the most important findings is that the relative population of bound water is drastically increased by the accumulation of trehalose. About 80% of the intracellular water in the trehalose-containing yeast cells is attributed to bound water. Similar results are obtained for trehalose-containing and -lacking model membranes prepared from dipalmitoylphosphatidylcholine (DPPC). Additionally, DSC measurements are carried out to examine effects of trehalose on the gel–liquid crystalline transition temperature of the DPPC membranes. On the basis of these results, a brief discussion is given about the role of trehalose as a stress tolerance-inducer in cells.

A variety of organisms such as brine shrimp cysts, certain nematode, and yeast can survive in the dry state, a phenomenon called anhydrobiosis.[1—4] These organisms are thought to acquire the stress tolerance by synthesizing a large amount of disaccharide, trehalose, in their intracellular media.[4] Similarly, there is much evidence to presume that other types of stress tolerance, such as high temperature- and freeze-tolerances, are also acquired by enhanced accumulation of intracellular trehalose.[5—10] These findings imply that trehalose plays a key role in stabilizing membranes and/or other macromolecular assemblies under extreme environmental conditions.

One mechanism by which trehalose (or other saccharides) stabilized membranes in the dry state is known as the water-replacement hypothesis,[11] meaning that trehalose is directly bound to polar head groups of phospholipid, probably via hydrogen bondings, in place of water. Binding of trehalose to the membrane surface causes a depression of the gel-liquid crystalline transition temperature well below that of the fully hydrated lipids. As a result of this, the dry membranes do not undergo the phase transition during rehydration, and thereby the leakage of intracellular contents is minimized. A variety of *in vitro* studies using reconstructed lipid bilayers have provided excellent evidence on this hypothesis.[1—4] Recently, it has been confirmed that this hypothesis is also valid in intact cells.[4,12] Those studies mainly focused on the direct hydrogen bonding of trehalose with phospholipid. However, it remains unclear how the other stress tolerances are mediated by intracellular trehalose.

Recently, Obuchi and co-workers have found that by heat shock treatment baker's yeast (*Saccharomyces cerevisiae*) simultaneously acquires different kinds of stress tolerances, such as high temperature-, high pressure-, and freeze-tolerances.[13—19] And their *in vivo* ^{1}H NMR and DSC data have shown that the intracellular water are highly structured as a response to heat shock: namely, an increase in unfrozen water and an immobilization of some portion of free water.[19] Consequently, they suggested that heat shock proteins play a role in serving a larger amount of hydration water to membrane and/or other macromolecular assemblies, and thereby such biological macromolecules are protected from denaturation. According to this hypothesis, structuring of the intracellular water is a key factor endowing cells with various stress-tolerances.

There is extensive evidence to believe that trehalose possesses the high ability of modifying water structure. According to our recent study,[20] trehalose has a larger amount of unfrozen water per glucose residue than the other oligosaccharides examined, and its bound water is highly immobilized on the basis of ^{17}O NMR mea-

surements of water in the aqueous solution of trehalose. In addition, the aqueous solution of trehalose exhibits anomalously high vitrification temperature[21] and high compressibility[22] compared with maltose or sucrose. In view of these findings, it is of great interest to investigate effects of trehalose on the intracellular water structure for a better understanding of its role as a stress protectant in cells.

In this paper, *in vivo* ^{1}H NMR results are first presented for trehalose-containing and -lacking yeast cells, which were prepared without any heat shock treatment. Next, we describe ^{1}H NMR results for trehalose-containing and -lacking model membranes. It is shown that the observation of T_2 relaxation time provides invaluable insight into the intracellular water structure. Combining the *in vivo* and *in vitro* results, it is demonstrated that trehalose drastically modifies the water structure in the yeast cells, resulting in an increase of bound water.

Experimental

Preparation of Yeast Samples. Commercial compressed yeast was provided from Asahi Chemical Industry Co., Ltd., Japan. Trehalose was extracted with 0.5 M trichloroacetic acid (M=mol dm^{-3}) and determined by the anthrone method[23] or high-pressure liquid chromatography. The trichloroacetic acid extract was applied to LC-9 with a SC1011 column (8×300 mm; Showa Denko, Japan) and eluted with distilled water. A refractive index detector was used. The compressed yeast cells contained 8.1% of trehalose in weight fraction to their dry solid. The cells were suspended in an appropriate amount of distilled water and subsequently lyophilized.

Trehalose-lacking yeast cells were prepared from cells in the logarithmic growth phases as follows.[7] Commercial baker's yeast was grown on a slant of YPD (0.5% yeast extract, 1% peptone, 2% dextrose, 0.5% KH_2PO_4, 0.3% $MgSO_4$) medium for 24 h at 30 °C. The cell suspension from the resulting slant was inoculated to 100 ml of YPD broth in a 500-ml Sakaguchi flask. After 24 h of shaking at a speed of 140 rpm at 30 °C, a fraction of the culture was transferred to the same medium contained in a jar fermenter. After 8 h of cultivation, logarithmic phase-cells were collected by centrifugation, followed by freezing at −20 °C and subsequent lyophilization.

The resulting lyophilized solids, obtained from both compressed and logarithmic phase-yeast cells, were used as NMR samples after the subsequent treatment of hydration or further drying. Highly hydrated samples were obtained by standing the lyophilized samples in a desiccator with appropriate humidity. Further drying was carried out in a desiccator containing anhydrous $CaSO_4$. In both cases of hydration and drying, different levels of water content were attained by changing the time for which the samples were standing in the desiccators in a range from one to seven days. Hereafter, the water content is defined as the weight fraction of the residual water to the dry solid (cells) obtained by evaporating the samples at 110 °C for 24 h in vacuo.

Preparation of Model Membranes. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma Chemical Co., Inc., St. Louis and trehalose from Nakarai Chemical Co., Inc., Tokyo. DPPC vesicles were prepared as follows. DPPC (0.0015 mol) was dispersed in 100 ml of water. After 30 min of shaking at 50 °C, the solution was freezed and finally lyophilized. Trehalose-containing vesicles were prepared in the same way except that DPPC (0.0015 mol) was first dispersed in the aqueous solution of trehalose (0.0015 mol). The molar ratio of trehalose to DPPC in the vesicles obtained is 1 : 1. The water content of the vesicles was determined as similar to the case of yeast.

NMR Measurements and Data Analysis. ^{1}H NMR measurements were performed with a JEOL GSX-270 spectrometer operating at 270.06 MHz. The spin–spin relaxation times (T_2) were determined using the standard Carr–Purcell method with Meiboom–Gill modification.[24] All measurements were made at 30 °C unless otherwise noted. It was found that the free induction decay (FID) of the protons in the yeast samples involves an extremely fast-relaxing component, resulting in a broad peak in the frequency domain. A similar fast-relaxing component ($T_2 \fallingdotseq 10$ ms) has been detected in the proton decay signals in muscle and assigned to the protons on macromolecules (membrane, proteins, and nucleic acids).[25] Here, in order to avoid sampling the signals of these protons, the initial part of FID was truncated by giving a fairly large value (70 ms) to the delay time at which sampling is to start after the radio frequency pulse has ended. Such a technical modification has no significant influence on the T_2 measurements of other slower-relaxing components, because their T_2 values are not less than 1 ms as will be described later.

In both cases of yeast and DPPC, the FID exhibited multi-exponential behavior as shown in Fig. 1, and thus was analyzed by using the following equation:

$$h_{xy}(t) = h_{xy}(0)\,[\sum a_j \exp\{-(t/T_{2j})\}] \qquad (1)$$

where $h_{xy}(t)$ is the transverse magnetization observed at

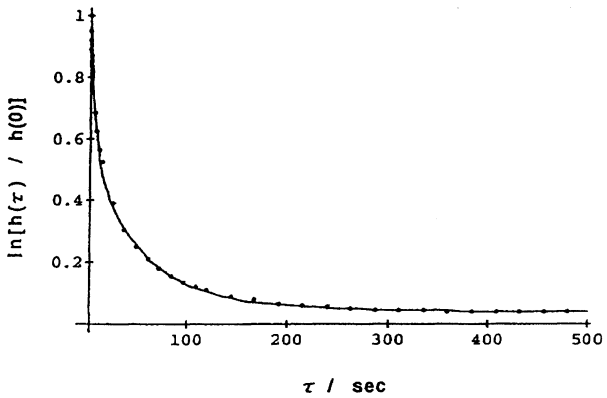

Fig. 1. T_2 relaxation behavior of water protons in the trehalose-lacking yeast sample with a water content of 3.89%. This figure is a direct output from a computer after the analysis of a least-squares fit was finished. The magnetization ratio $h(t)/h(0)$ is plotted as a function of time after 90° pulse in Carr–Purcell–Meiboom–Gill sequence. The solid line represents the relaxation behavior of the major component in the multi exponential fit. In this case, Eq. 1 is given as follows: $h(t)/h(0) = 0.06\exp(-t/1086) + 0.45\exp(-t/5.5) + 0.49\exp(-t/53)$.

time t after the 90° pulse, and a_j is fractional population of the component j having the relaxation time T_{2j}. We used the computer program "Mathematica ver. 2.1", produced by Wolram Research Inc., 1992, in order to find least-squares fits to a given set of the observed data $(h_{xy}(t)/h_{xy}(0), t)$. After a process of trial and error, we found that the expansion using two or three sets of a_j and T_{2j} are sufficient to obtain a better curve fitting as shown in Fig. 1. This is consistent with the results of earlier NMR relaxation studies on water protons in muscles.[26—28]

DSC Measurements. DSC thermograms were obtained using a Seiko DSC-20, equipped with a Seiko SSC/580 thermal controller. Approximately 3-10 mg of the DPPC membrane prepared above was placed in a sealed silver pan with an empty pan as reference. The observed temperature range was 0—100 °C and the heating rate 5 °C min^{-1}.

Results

Representative ^{1}H NMR spectra of the trehalose-lacking yeast cells are shown in Fig. 2, where the spectrum C corresponds to that of the lyophilized sample which had not undergo the post-treatment of hydration or drying and exhibited the water content of 6%. When the water content is lower than 6%, only one resonance is observed at the center position. On the other hand, in more hydrated samples, a new resonance appears at about 5 ppm and its intensity becomes large with increasing water content. Similar results were also obtained for the trehalose-containing yeast cells (data not shown). For convenience of the later discussion, the center and downfield-side resonances are denoted as peaks 1 and 2, respectively. The occurrence of such a resonance shift indicates that the water protons in the yeast cells are distributed between at least two different compartments.

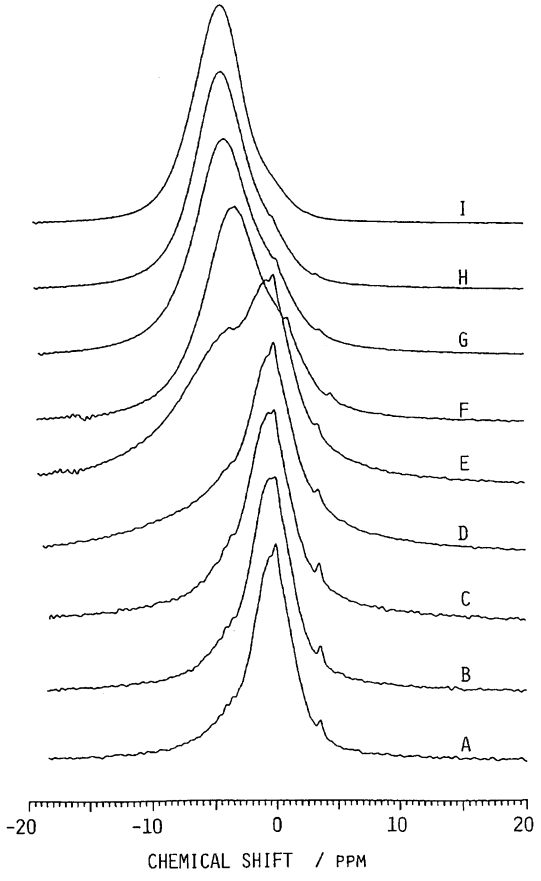

Fig. 2. ^{1}H NMR spectra of water in the trehalose-lacking yeast samples. The water contents of the samples for spectra A-I are 3.65, 4.39, 5.88, 7.60, 10.03, 15.80, 18.73, 25.10, and 34.59%, respectively. All the peak intensities are normalized in arbitrary unit.

The T_2 relaxation process of peak 1 was observed without interference from peak 2 only when the water content was less than 10%. The resulting decay curve (Fig. 1) was decomposed into two relaxing components. The T_2 value of the faster-relaxing component was determined to be around 5 ms, and that of the other to be around 50 ms. Hereafter, these components are abbreviated as A- and B-components, respectively. Furthermore, from measurements using highly hydrated samples (water content; >10%), the T_2 values for peak 2 was determined to be nearly equal to that for the A-component of peak 1. Figure 3 shows the water content dependence of the T_2 relaxation times for the A-components of peaks 1 and 2. Two notable features are pointed out. First, in the range of low water content (<10%), the water protons in the trehalose-containing yeast cells possesses shorter relaxation times than those in the trehalose-lacking ones. According to the accumulated studies on the T_2 relaxation process of water proton in various biological systems,[29] the relaxation time is expected to decrease monotonously with an increase in the motional correlation time. Thus, the present finding demonstrates that the cell waters contributing to the A-component are more immobilized in

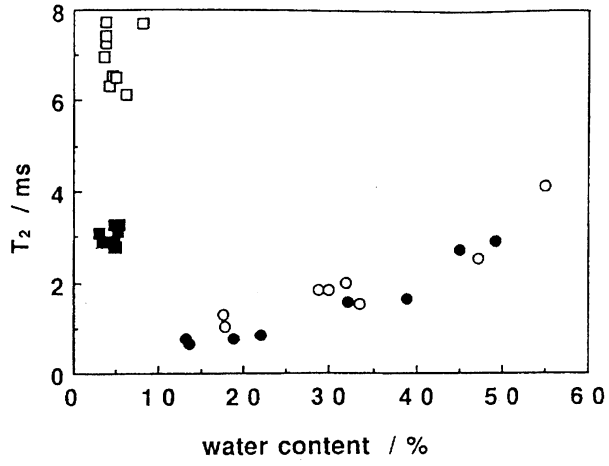

Fig. 3. Water content dependence of the T_2 relaxation time of water proton in the yeast samples. The open and solid symbols indicate the data for the trehalose-lacking and -containing samples, respectively. The squares and circles represent the data obtained from peaks 1 and 2 (Fig. 2), respectively.

the presence of trehalose. Similar results were obtained for the B-component. The T_2 relaxation data for the trehalose-lacking and -containing samples are summarized in Tables 1 and 2, respectively. Second, in the highly hydrated samples (water content; 10%<), the T_2 relaxation time for peak 2 increases with increasing water content, indicating that the mobility of water molecules contributing to peak 2 increases with water content.

Tables 1 and 2 also summarize the fractional populations of water molecules contributing to the A- and B-components of peak 1. One surprising finding is that the relative population between the two components drastically changes according to whether the yeast cells contain trehalose or not. In earlier ^{1}H NMR studies,[26,27] a relaxing component with T_2 of ca. 5 ms was also observed for water protons in muscle, and assigned to the protons of water molecules tightly bound to macromolecules. And a slower-relaxing component with T_2 of ca. 50 ms was assigned to the protons of the residual cell water molecules. According to this classification, a large fraction (0.7—0.8) of the cell waters in the trehalose-containing yeast cells can be regarded as bound water. Figure 4 shows the data for another series of experiments using different yeast samples, again indicating that the component B water is much less populated in the trehalose-containing yeast cells than in the trehalose-lacking ones.

Table 1. The Water Content Dependence of the Proton T_2 Relaxation Times and the Cell Water Structure in the Trehalose-Lacking Yeast cells

Water content[a]	Component[b]	Population[c]	T_2[d]
%			ms
3.89	A	0.49	5.5
	B	0.51	54.
	C	0.0	
6.16	A	0.46	5.0
	B	0.54	56.
	C	0.0	
6.25	A	0.51	5.5
	B	0.49	55.
	C	0.0	

a) Weight fraction of the residual water to the dry cells. b) Components A and B correspond to the bound and intermediate waters, respectively. c) Maximal errors, accompanied by the curve fitting analysis (see Eq. 1), are ±0.01. d) Relative experiental errors are ±10%.

Table 2. The Water Content Dependence of the Proton T_2 Relaxation Times and the Cell Water Structure in the Trehalose-Containing Yeast Cells

Water content[a]	Component[b]	Population[c]	T_2[d]
%			ms
1.91	A	0.77	3.3
	B	0.24	38.
	C	0.0	
2.15	A	0.76	3.3
	B	0.24	33.
	C	0.0	
2.67	A	0.77	3.3
	B	0.23	33.
	C	0.0	
3.53	A	0.77	3.5
	B	0.23	37.
	C	0.0	

a) Weight fraction of the residual water to the dry cells. b) Components A and B correspond to the bound and intermediate waters, respectively. c) Maximal errors, accompanied by the curve fitting analysis (see Eq. 1), are ±0.02. d) Relative experiental errors are ±10%.

In contrast to the case of yeast, the water protons in DPPC model membranes exhibited a single peak irrespective of their hydration levels. And for the trehalose-lacking samples, the third relaxing component with T_2 of ca. 100 ms was detected as shown in Table 3. The earlier studies has assigned such protons with T_2>100 ms to extra cellular water,[26,27] which are hereafter denoted as component C. For the multilamellar membranes, the classification of intra- and extra-cellular waters is inappropriate. It seems to be reasonable that the component C water is assigned to free water located fully distant from the membrane surface. In the trehalose-containing samples, the T_2 decay curve could be again decomposed into two relaxing components and each fractional population was determined. From Tables 3 and 4, it is confirmed that trehalose has significant effects on properties of water in the model membranes as well. As similar to the case of yeast, the population of bound water is drastically increased by the addition of trehalose. It should be noted that free water completely disappears in the trehalose-containing membranes even in highly hydrated samples.

The gel–liquid crystalline transition temperature, T_m, of the DPPC membrane is shown in Fig. 5, where

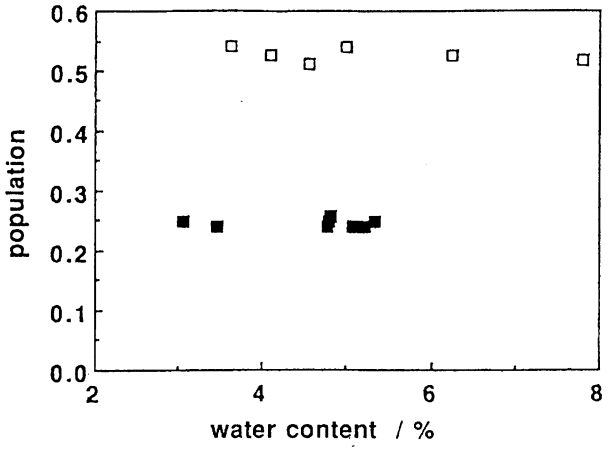

Fig. 4. Water content dependence of the fractional population of intracellular water belonging to component B. The open and solid symbols indicate the data for trehalose-lacking and -containing samples.

Table 3. The Water Content Dependence of the Proton T_2 Relaxation Times and the Water Structute in the Trehalose-Lacking DPPC Membranes

Water content		Component[a]	Population[c]		T_2[d]
%[b]	Molar ratio[c]		Relative[d]	Absolute[e]	ms
7.77	3.17	A	0.25	0.79	6.0
		B	0.75	2.38	20.
		C	0.0	0.0	
12.70	5.18	A	0.15	0.76	7.0
		B	0.83	4.30	36.
		C	0.02	0.11	100.
18.43	7.51	A	0.23	1.73	8.0
		B	0.74	5.56	34.
		C	0.03	0.23	110.
20.65	8.42	A	0.24	1.98	8.0
		B	0.73	6.15	32.
		C	0.04	0.30	100.
21.66	8.83	A	0.23	1.99	8.0
		B	0.75	6.58	34.
		C	0.03	0.22	100.

a) Components A, B, and C corresponds to the bound, intermediate, and free waters, respectively. b) Weight fraction of the residual water to the dry solid. c) Molar ratio of the residual water to the DPPC molecules (H_2O/DPPC). d) Relative popolations. Maximal errors, accompanied by the curve fitting analysis (see Eq. 1), are ±0.01. e) Absolute number of water molecules belonging to each component. f) Relative experimental errors are ±10%.

Table 4. The Water Content Dependence of the Proton T_2 Relaxation Times and the Water Structute in the Trehalose-Containing DPPC Membranes

Water content		Component[a]	Population		T_2
%[b]	Molar ratio[c]		Relative[d]	Absolute[e]	ms
8.02	4.80	A	0.87	4.17	6.0
		B	0.13	0.62	29.
		C	0.0	0.0	
9.83	5.88	A	0.82	4.79	7.0
		B	0.19	1.10	28.
		C	0.0	0.0	
17.07	10.21	A	0.79	8.07	8.0
		B	0.21	2.14	31.
		C	0.0	0.0	
18.07	10.81	A	0.80	8.59	8.0
		B	0.21	2.22	30.
		C	0.04	0.30	100.
18.56	11.10	A	0.78	8.66	8.0
		B	0.22	2.44	33.
		C	0.0		

a) Components A, B, and C corresponds to the bound, intermediate, and free waters, respectively. b) Weight fraction of the residual water to the dry solid. c) Molar ratio of the residual water to the DPPC molecules (H_2O/DPPC). d) Relative popolation. Maximal errors, accompanied by the curve fitting analysis (see Eq. 1), are ±0.02. e) Absolute number of water molecules belonging to each component. f) Relative experimental errors are ±10%.

the water content H_2O/DPPC is expressed as a molar ratio of the residual water to DPPC. In the range of water content from one to five, the transition temperature of the trehalose containing-DPPC membrane seems to be lower than that of the trehalose-lacking one, although the number of the data points for the former is not necessarily sufficient. In the more hydrated samples, there is no apparent difference in T_m between both systems.

Discussion

The water molecules in the yeast cells studied seem to be distributed between at least two compartments, because two proton signals (peaks 1 and 2) were ob-

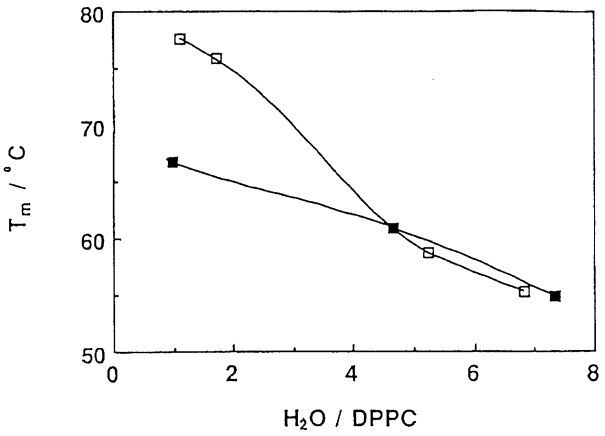

Fig. 5. Water content dependence of the gel-liquid crystalline transition temperature of the DPPC membranes. Water content is expressed as the molar ratio of the residual water to DPPC molecules. The open and solid symbols indicate the data for the trehalose-lacking and -containing samples.

served as shown in Fig. 2. The present study alone can not provide an unambiguous explanation for the origin of such a peak shift. However, the following facts may be helpful to consider the origin of peak 2. First, the relative intensity of peak 2 to that of peak 1 increased with increasing water content, and their proton exchange rate was fully slow compared with the characteristic time scale of NMR spectroscopy. Second, the highly hydrated samples, for which peak 2 was observed, were prepared by the post-treatment of lyophilized solids as described above. Combining these facts, it seems likely that peak 2 is assigned to the protons of water molecules bound to extra cellular surfaces of the yeast cells. Therefore, we will focus mainly on the relaxation data for peak 1 in the subsequent discussion.

One of the most important findings in this study is that the fractional population of bound water (component A) in the yeast cells is markedly increased by the accumulation of intracellular trehalose. Similarly, the population of bound water in the DPPC membranes is drastically increased by the addition of trehalose. The resulting ratio (4 : 1) of components A to B are in fairly good agreement with that observed for the yeast samples, although the observed range of water content is different between the yeast and DPPC samples. These results indicate that the average mobility of the residual water is significantly lowered by the addition of trehalose in both *in vivo* and *in vitro* systems, implying the molecular mechanism of trehalose-mediated stress tolerance.

As described in the introductory remarks,[1—4] the water-replacement hypothesis is a well known mechanism for desiccation tolerance. If this is true, the gel–liquid crystalline transition temperature T_m would be affected by the addition of trehalose. As shown in Fig. 5, the T_m value of the trehalose-containing DPPC membrane is likely to be lower than that of the trehalose-lacking membrane in an extremely dehydrated region where the H_2O/DPPC ratio is less than five, but in the more hydrated region the T_m values of both systems are nearly equal. These trends are consistent with a previous report by Tsvetkov et al.,[30] who indicated that the transition temperature of freeze–dried DPPC membrane containing trehalose is lower by about 10 °C than that of trehalose-lacking membrane, but in the fully hydrated state both systems exhibit the same transition temperature. The water-replacement hypothesis may be thus effective only under the extremely dehydrated conditions.

According to the present ^{1}H NMR data for the DPPC membrane, the higher population of bound water is observed in a range beyond the H_2O/DPPC ratio≒5. Thus, if such a population change, in going from the trehalose-containing to -lacking samples, is responsible for a function of trehalose as a protecting agent, one must assume another mechanism. As a biological membrane containing sugar is dehydrated, free water would be first dissipated, leaving a concentrated mixture composed of sugar and water. If the resulting mixture is vitrified, the membrane would be capsulized, leading to protection of the membrane structure. Recently, the validity of such a mechanism has been pointed out by many workers.[31—33] Although the present ^{1}H NMR results does not necessarily provide direct evidence on the occurrence of vitrification, its possibility may be partially supported by the fact that the average mobility of the residual water is significantly lowered by the addition of trehalose.

Conclusion

In this study, trehalose-lacking yeast was prepared from cells in the logarithmic growth phases, and their ^{1}H NMR results were compared with those for compressed yeast containing trehalose. It is not clear whether both types of samples are different from each other only in trehalose composition or not. However, one of the authors (A. K) has already demonstrated that there is correlation between the freeze tolerance of yeast and its growth phases giving cells with the different amounts of intracellular trehalose.[7] Thus, the comparison of both types of samples has a biological significance. With the aid of the results for the DPPC membrane, it is reasonable to be concluded that the higher population of bound water observed in compressed yeast is induced by the accumulation of intracellular trehalose.

The authors thank Dr. H. Nagase of Hoshi University for inspiring discussions about the DSC data, and Professor Y. Yamamoto for help in the NMR experiments.

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