Supporting Information

for

Hydration and mobility of trehalose in aqueous solution

Louise Revsbech Winther, Johan Qvist and Bertil Halle

S1. PREPARATION AND CHARACTERIZATION OF DEUTERATED TREHALOSE

Catalytic Hydrogen Exchange. Trehalose was deuterated using the hydrogen-exchange catalyst Raney nickel (a fine-grained Ni-Al alloy) according to the protocol of Koch and Stuart.[1] In D_2O solution, Raney nickel catalyzes the H → D exchange of hydrogen atoms bound to carbon atoms with a geminal hydroxyl group. Trehalose contains 10 such hydrogens, bound to 8 carbon atoms.

About 50 ml Raney nickel sediment from a H_2O suspension (Raney 4200 slurry, Aldrich) was washed 3 times with an equal volume D_2O (99.8 atom%, Cortecnet). The crystal water and hydroxyl groups of α,α-trehalose dihydrate ($\geq$99.0 % purity, Fluka) were H→D exchanged by dissolving 15 g trehalose in 80 ml D_2O and evaporating the solvent at 50 °C. Dried trehalose and washed Raney nickel were then redissolved together in D_2O to 300 ml and the suspension was refluxed on a water bath at 97 °C. Samples were taken every 1 – 2 h to check the deuteration level by NMR. After 11 h the reaction mixture was cooled to room temperature and filtered through 2 layers of filter paper and then through 0.2 μm syringe filters. Finally, 40 μl 1 % sodium azide solution was added to prevent bacterial growth.

Ion Exchange. To remove metal ions, in particular paramagnetic Ni^{2+} ions which might affect the relaxation measurements (see Sec. S2), the filtered solution was passed through a Chelex 100 ion-exchange column (Bio-Rad). Prior to use, the basic Chelex resin was rinsed 3 times with 0.5 M sodium acetate at pH 4.7 and then 5 times with MilliQ water. The resulting pH of the resin was 7 – 8. The deuterated trehalose solution was slowly pumped through the 1×10 cm Chelex column, which was regenerated after every 50 ml solution to eliminate bound metal ions. To regenerate the Chelex, it was washed with two bed volumes 1 M HCl, then with at least 5 bed volumes MilliQ water, then with 2 bed volumes 1 M NaOH, and finally flushed with water for 5 – 10 min.[2] After Chelex treatment, the solution was again passed through 0.2 μm syringe filters.

Crystallization. To obtain trehalose crystals, the solution was first dried at 50 °C, yielding, after several days, a rubbery syrup. To back-exchange D_2O and labile hydroxyl deuterons, the syrup was dissolved in MilliQ H_2O and left to dry at 50 °C. A 1:4 (v/v) water-ethanol crystallization solvent was then added (10 ml solvent per gram dried syrup).[3,4] The crystals that had formed on the glass surface after a few days were removed by brief heating and were then gently washed under suction with small amounts of water-ethanol mixture and dried at 50 °C. In order to remove the last D_2O from the crystals, they were crushed into a powder and dried under vacuum for several days.

Anion Exchange Chromatography. To test for saccharide isomers that may form as by-products in the Raney nickel deuteration process,[5] the crystalline product was examined with ion chromatography. To convert the sample to monosaccharide form, it was hydrolyzed in 4 % H_2SO_4 for 3 h and neutralized with $Ba(OH)_2$. The solution was then pumped through a CarboPac PA10 anion-exchange column (Dionex), kept basic with NaOH, and the eluate was monitored with pulsed amperometric detection. As expected, the chromatogram exhibits a dominant peak at the same elution time as glucose (Fig. S1). The slightly shorter elution time as compared to the glucose reference is presumably a H/D isotope effect. No other monosaccharides are present at detectable levels in the sample (Fig. S1). The two minor peaks with short elution times may be ethanol (from the crystallization solvent) and residual ions from the catalyst. The chromatographic characterization was performed on crystals from a batch that had only been dried briefly at 50 °C. The trehalose batch used for all NMR relaxation measurements was dried more exhaustively (by crushing the crystals and leaving them in a vacuum for a few days), so most of the ethanol seen in the chromatogram should have evaporated in this batch. This expectation was confirmed by the fact that the ^1H and ^{13}C NMR spectra (see below) revealed no trace of ethanol or any other organic impurity.

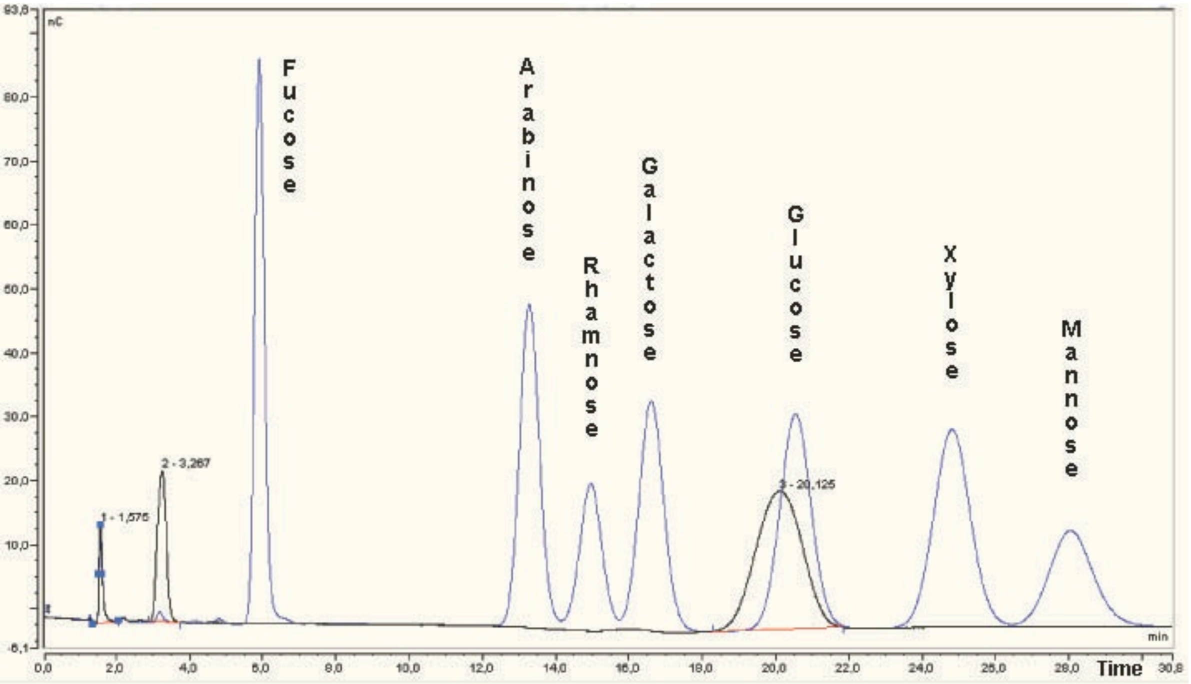

Figure S1. Anion exchange chromatogram of hydrolyzed trehalose deuteration product (black trace) and several monosaccharide references (blue trace).

^{1}H NMR spectroscopy. The trehalose deuteration level was assessed by ^{1}H NMR using a Varian VnmrS Direct-Drive spectrometer operating at 600 MHz. The proton peaks were assigned using the 2D [^{1}H,^{13}C]-HSQC spectrum for trehalose from the BRMB database[6] and published ^{13}C assignments.[7] The overlaid NMR spectra from partially deuterated and fully protonated trehalose are shown in Fig. S2. The NMR sample was recorded from the reaction mixture prior to removal of Ni^{2+} by anion exchange. The solvent is D_2O so the ^{1}H peaks are produced by the non-exchanged hydrogens in trehalose. The deuteration level ξ_k at carbon number k (Fig. S3) was obtained as

$$\xi_k \;=\; 1 - \frac{I_k}{I_1} \tag{S1}$$

where I_k is the integrated intensity of the peak assigned to protons bound to carbon k. The intensities were normalized with the intensity I_1 of carbon 1, which does not have a geminal hydroxyl group and hence should not be deuterated at all.

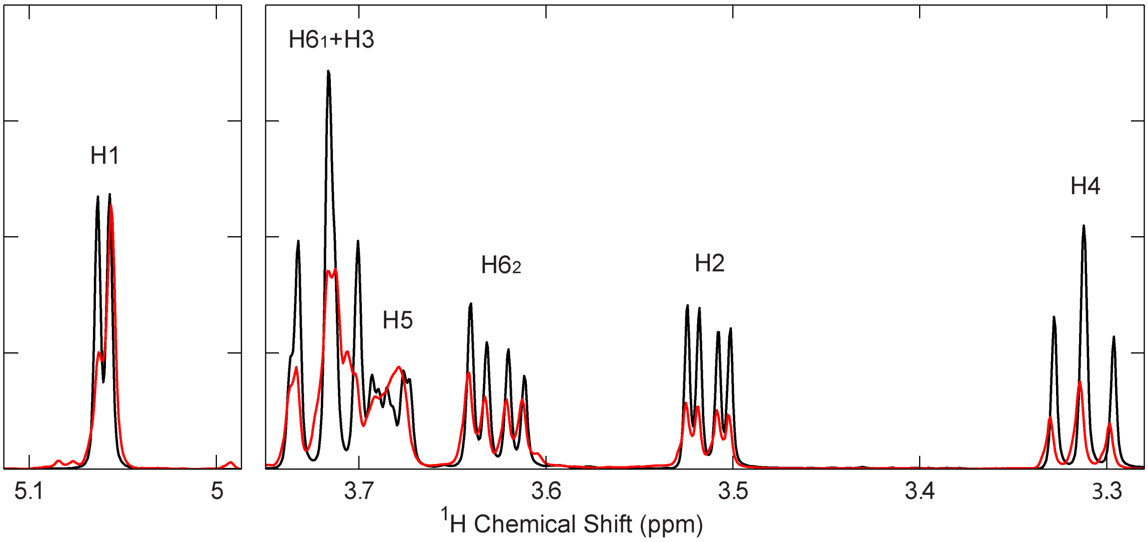

Figure S2. 600 MHz ^{1}H NMR spectra of trehalose reference (black trace) and partially deuterated trehalose (red trace), both in D_2O at 25 °C. Peak assignments use the carbon numbering in Fig. S3.

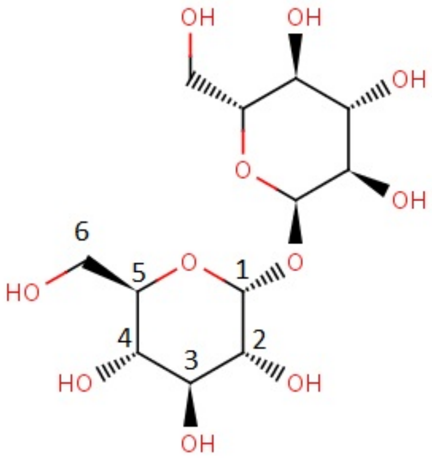

Figure S3. Carbon numbering in trehalose.

The deuteration levels thus obtained are $\xi_2 = 0.51$, $(\xi_3 + \xi_{6_1})/2 = 0.24$, $\xi_4 = 0.56$, $\xi_5 = 0$ (as expected) and $\xi_{6_2} = 0.22$. The average degree of deuteration in the 5 positions with a geminal hydroxyl group is thus 0.35, corresponding to 3.5 deuterons per trehalose molecule.

S2. PARAMAGNETIC RELAXATION ENHANCEMENT

Any Ni^{2+} ions that remain in our deuterated trehalose preparation despite the Chelex treatment would, on account of their two unpaired electrons, increase the water-^{17}O relaxation rate R_1. To demonstrate that this paramagnetic relaxation enhancement (PRE) is negligibly small, we analyzed a small sample from the most concentrated (1.47 M) trehalose solution used for ^{17}O relaxation measurements. After 200-fold dilution, this sample was analyzed by inductively coupled plasma sector field mass spectrometry (performed at ALS Analytica, Luleå, Sweden), yielding a Ni(II) concentration of 12.9 μM in the undiluted sample. Since the Ni(II) concentration is proportional to the trehalose concentration it is lower in the other solutions used for ^{17}O R_1 measurements. Because of the low concentration we can safely use the dilute-regime approximation, according to which the PRE is given by[8]

$$R_1^{\mathrm{PRE}} = \frac{f_{\mathrm{Ni}}}{\tau_{\mathrm{Ni}} + T_{1,\mathrm{Ni}}} \tag{S2}$$

where f_{Ni} is the fraction of all water molecules that are directly coordinated to a Ni^{2+} ion, τ_{Ni} is the mean residence time of these water molecules and $T_{1,\mathrm{Ni}}$ is their intrinsic ^{17}O longitudinal relaxation time (in the bound state). For the 1.5 M trehalose solution, $f_{\mathrm{Ni}} = 6 \times 12.9 \times 10^{-6} / 38.5 = 2.01 \times 10^{-6}$ since each Ni^{2+} ion coordinates 6 water molecules and the total water concentration in the 1.47 M trehalose solution is 38.5 M. The residence time is $\tau_{\mathrm{Ni}} = 32$ μs at 298 K[9] (at lower temperatures, τ_{Ni} is longer so the PRE is smaller). To obtain an upper bound for the PRE, we set $T_{1,\mathrm{Ni}} = 0$. From eq S2 we thus obtain $R_1^{\mathrm{PRE}} < 0.063\ \mathrm{s}^{-1}$, which is 3 – 5 orders of magnitude smaller than the ^{17}O R_1 (140 – 3300 s^{-1} in our temperature range) for bulk water. We thus conclude that the effect of residual Ni(II) is negligible at all temperatures and trehalose concentrations examined here.

S3. TREHALOSE CONCENTRATION DETERMINATION

^{13}C NMR Spectroscopy. The trehalose concentration in the solutions used for ^{2}H and ^{17}O relaxation measurements was determined by natural-abundance ^{13}C NMR using a Varian VnmrS Direct-Drive spectrometer operating at 150.8 MHz. To allow the concentration to be accurately determined from integrated peak intensities, the ^{13}C spectrum (Fig. S4) was recorded with inverse gated broadband proton decoupling (during ^{13}C signal acquisition only) using the WALTZ-16 pulse sequence.[10] To obtain an acceptable signal-to-noise ratio with 8 or 16*k* transients and 7 or 15 s recycle delay, the ^{13}C relaxation rate was increased by performing the measurements at 4 °C and by adding the paramagnetic relaxation agent $MnCl_2$ to a concentration of 5 mM.

To obtain absolute concentrations, isobutanol was added to the solution at an accurately known concentration C_{ref} (in the range 0.3 – 0.5 M). The trehalose concentration C was deduced by comparing the integrated intensity I of a trehalose peak with the intensity I_{ref} of an isobutanol peak:

$$C \;=\; C_{\text{ref}}\,\frac{n}{2}\,\frac{I}{I_{\text{ref}}} \tag{S3}$$

where $n = 1$ (peak iB2) or 2 (iB3) is the number of equivalent carbons contribution to the isobutanol peak and the factor 2 takes into account that each trehalose peak represents two equivalent carbons, one in each glucose ring. The reported trehalose concentration is the average of the four values obtained from two trehalose peaks (1 and 3) and two isobutanol peaks (iB2 and iB3).

In this way, the concentration was determined for the three stock solutions ($C = 1.47 \pm 0.01$ M, 1.31 ± 0.02 M and 0.249 ± 0.005 M) used to prepare all solutions for ^{2}H and ^{17}O relaxation measurements. For the other solutions, the concentration was calculated from the known dilution factors. Taking into account the error from the ^{13}C intensity measurements as well as a pipetting error of 0.5 %, we estimate that the trehalose concentrations are accurate to 1 – 2 %.

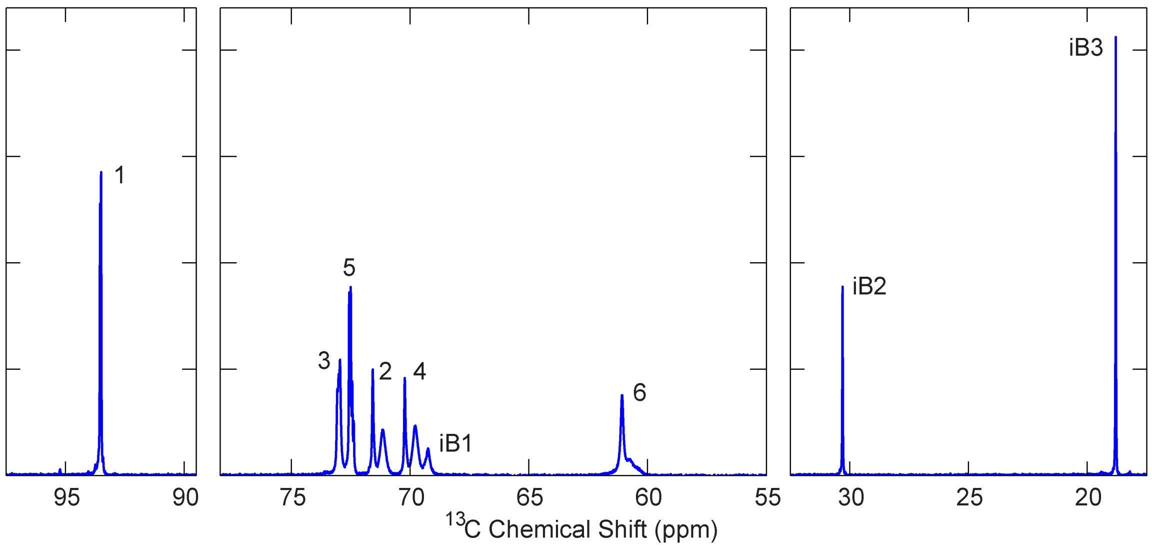

Figure S4. 151 MHz natural-abundance ^{13}C NMR spectrum of 1.47 M partially deuterated trehalose (peaks assigned with carbon numbering as in Fig. S3) and isobutanol (assigned peaks iB1 – iB3) in H_2O with 5 mM $MnCl_2$ at 4 °C.

Molar Composition. For the analysis of the water ^{17}O relaxation data, it is convenient to express the trehalose concentration in terms of the water/trehalose mole ratio N_W, which is obtained from the molar concentration C as

$$N_W = \frac{1 - C\,V_T}{C\,V_W} \tag{S4}$$

where $V_W = 18.05$ cm^3 mol^{-1} (at 20 °C) is the molar volume of bulk H_2O and V_T is the apparent partial molar volume of trehalose. For the latter, we used the value 206.85 cm^3 mol^{-1} (at the temperature, 20 °C, where C was determined) at infinite dilution.[11] Taking into account the weak concentration dependence[11] of V_T would only change N_W by 0.5 % at the highest concentration used here.

S4. CONTROL EXPERIMENTS ON EMULSION SAMPLES

To check if the interface of the emulsion droplets has any effect on the relaxation rate, we measured the trehalose ^{2}H R_1 (Fig. S5) and the water ^{17}O R_1 (Fig. S6) at 20.0 °C in trehalose solutions that were, or were not, contained in emulsion droplets. No significant differences could be detected.

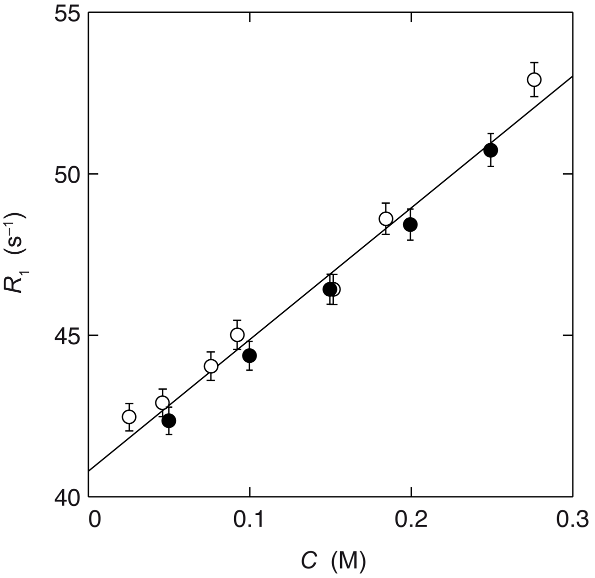

Figure S5. Trehalose ^{2}H relaxation rate R_1 versus trehalose concentration at 20.0 °C for emulsified (solid symbols) and non-emulsified (open symbols) solutions.

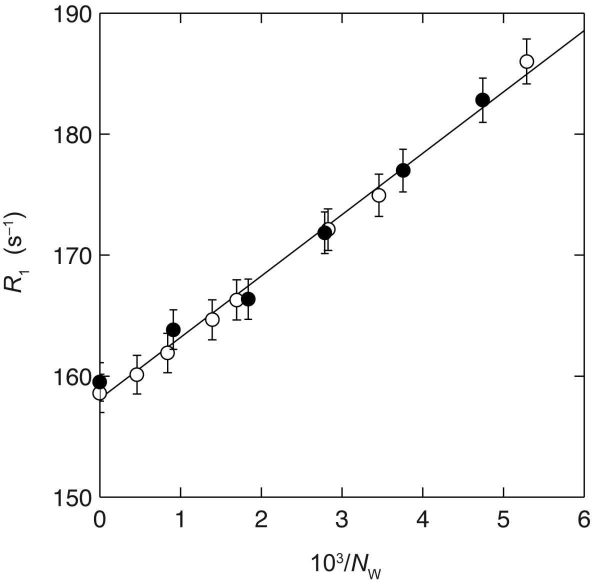

Figure S6. Water ^{17}O relaxation rate R_1 versus trehalose concentration (expressed in terms of the water/trehalose mole ratio N_W) at 20.0 °C for emulsified (solid symbols) and non-emulsified (open symbols) solutions.

S5. TEMPERATURE-DEPENDENT HYDRATION DYNAMICS

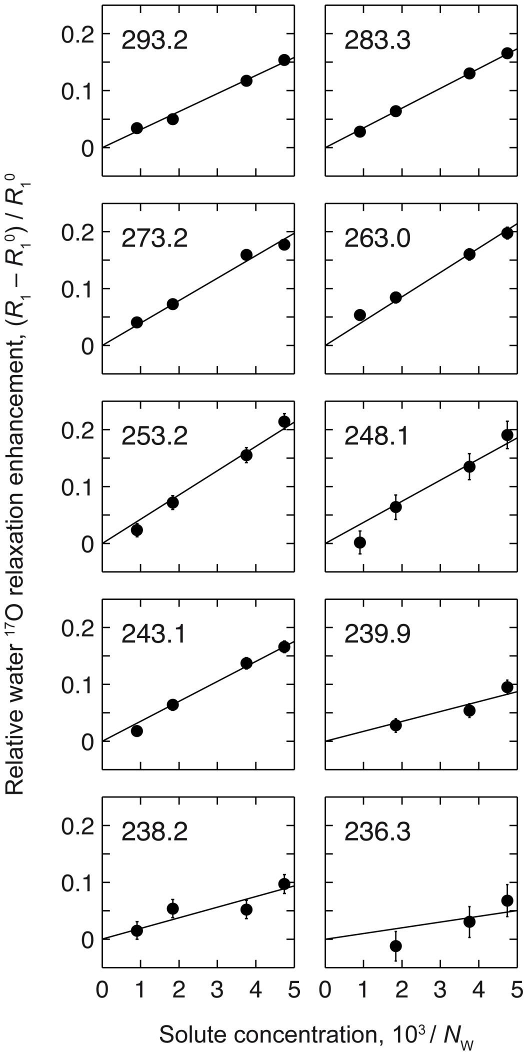

Figure S7. Relative water ^{17}O relaxation enhancement $(R_1 - R_1^0)/R_1^0$ at ten temperatures versus trehalose concentration, expressed in terms of the water/trehalose mole ratio N_W, in the independent hydration regime. At each temperature, the relative error in R_1 was iteratively adjusted to yield χ^2_{red} = 1 in the linear fit, which was forced to pass through the origin.

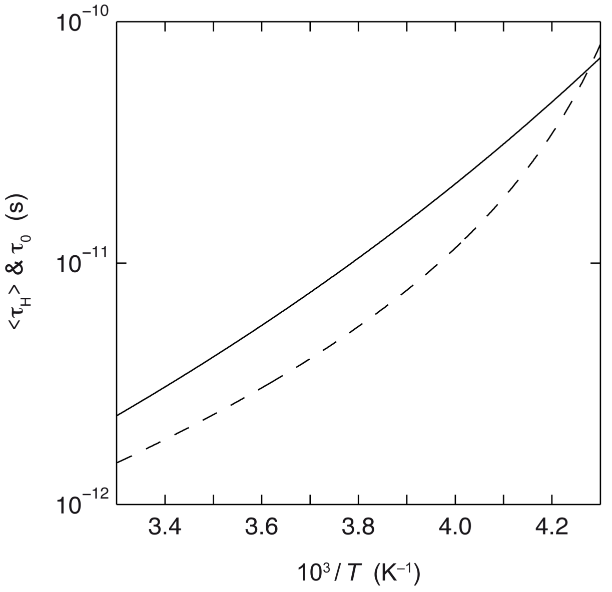

Figure S8. Arrhenius plot of the temperature dependence of the bulk water correlation time τ_0 (dashed) and the average hydration-layer correlation time $\langle\tau_H\rangle$ (solid).

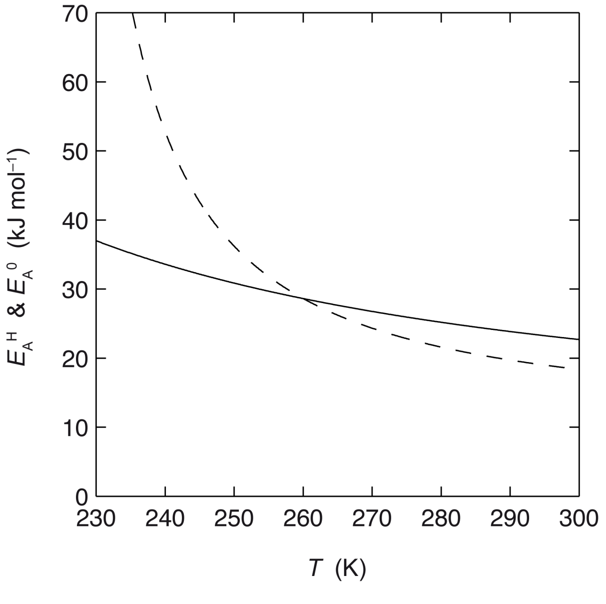

Figure S9. Temperature dependence of the apparent activation energy for water rotation in bulk H_2O (dashed) and in the hydration layer of trehalose (solid).

S6. THz DIELECTRIC ABSORPTION

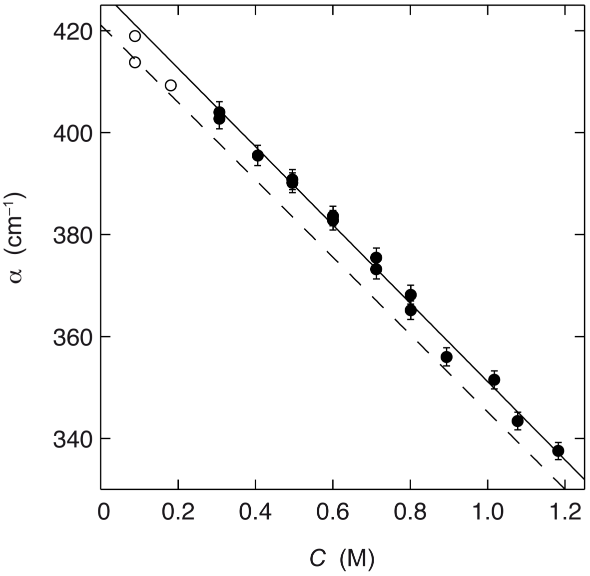

Figure S10. Dielectric absorption coefficient at 2.1 – 2.8 THz versus trehalose concentration in aqueous solution at 293.2 K.[12] The solid line resulted from a linear fit to the data above 0.2 M (solid symbols). The dashed line represents the ideal (no solvent perturbation) limit. The slopes (in units of cm^{-1} M^{-1}) are –76.8 ± 1.8 (solid line) and –75.9 (dashed line).

S7. PROTEIN STABILIZATION BY TREHALOSE

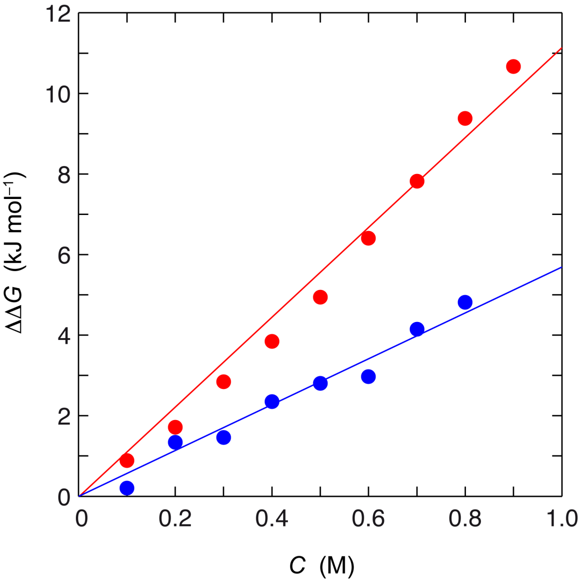

Figure S11. Stabilization free energy, $\Delta\Delta G = \Delta G(C) - \Delta G(0)$, at 20 °C and pH 2.8 (red) or 5.5 (blue) for thermal denaturation of ribonuclease A versus trehalose concentration C.[13] The m value derived from the slope is 11.2 kJ mol^{-1} M^{-1} at pH 2.8 and 5.7 kJ mol^{-1} M^{-1} at pH 5.5.

References

(1) Koch, H. J.; Stuart, R. S. *Carbohydr. Res.* **1977**, *59,* C1-C6.

(2) http://structbio.vanderbilt.edu/chazin/wisdom/labpro/chelex.html.

(3) Chi, Z.; Liu, J.; Zhang, W. *Enzyme Microbiol. Technol.* **2001**, *28,* 240-245.

(4) Chi, Z.; Wang, J.-M.; Chi, Z.-M.; Ye, F. *J. Ind. Microbiol. Biotechnol.* **2010**, *37,* 19-25.

(5) Balza, F.; Cyr, N.; Hamer, G. K.; Perlin, A. S. *Catbohydr. Res.* **1977**, *59,* C7-C11.

(6) http://www.bmrb.wisc.edu.

(7) Asano, N.; Matsui, K.; Takeda, S.; Kono, Y. *Carbohydr. Res.* **1993**, *243,* 71-78.

(8) Swift, T. J.; Connick, R. E. *J. Chem. Phys.* **1962**, *37,* 307-320.

(9) Bechtold, D. B.; Liu, G.; Dodgen, H. W.; Hunt, J. P. *J. Phys. Chem.* **1978**, *82,* 333-337.

(10) Shaka, A. J.; Keeler, J.; Freeman, R. *J. Magn. Reson.* **1983**, *53,* 313-340.

(11) Banipal, P. K.; Banipal, T. S.; Lark, B. S.; Ahluwalia, J. C. *J. Chem. Soc., Faraday Trans.* **1997**, *93,* 81-87.

(12) Heyden, M.; Bründemann, E.; Heugen, U.; Niehues, G.; Leitner, D. M.; Havenith, M. *J. Am. Chem. Soc.* **2008**, *130,* 5773-5779.

(13) Xie, G. F.; Timasheff, S. N. *Biophys. Chem.* **1997**, *64,* 25-43.