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Heterogeneous Ordered-Disordered Structure of the Mesodomain in Frozen Sucrose-Water Solutions Revealed by Multiple Electron Paramagnetic Resonance Spectroscopies

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Abstract

The microscopic structure of frozen aqueous sucrose solutions, over concentrations of 0–75% (w/v), is characterized by using multiple continuous-wave and pulsed electron paramagnetic resonance (EPR) spectroscopic and relaxation techniques and the paramagnetic spin probe, TEMPOL. The temperature dependence of the TEMPOL EPR lineshape anisotropy reveals a mobility transition, specified at 205 K in pure water and 255 ± 5 K for >1% (w/v) added sucrose. The transition temperature is $\gg T_g$, where T_g is the homogeneous water glass transition temperature, which shows that TEMPOL resides in the mesoscopic domain (mesodomain) at water-ice crystallite boundaries, and that the mesodomain sucrose concentrations are comparable at >1% (w/v) added sucrose. Electron spin echo envelope modulation (ESEEM) spectroscopy of TEMPOL- $^2\text{H}_2$ -sucrose hyperfine interactions also indicates comparable sucrose concentrations in mesodomains at >1% (w/v) added sucrose. Electron spin echo (ESE) – detected longitudinal and phase memory relaxation times (T_1 and T_M , respectively) at 6 K indicate a general trend of increased mesodomain volume with added sucrose, in three stages: 1–15, 20–50, and >50% (w/v). The calibrated TEMPOL concentrations indicate that the mesodomain volume is less than the predicted maximally freeze-concentrated value [80 (w/w); 120% (w/v)], with transitions at 15–20% and 50% (w/v) starting sucrose. An ordered sucrose hydrate phase, which excludes TEMPOL, and a disordered, amorphous sucrose-water glass phase, in which TEMPOL resides, are proposed to compose a heterogeneous mesodomain. The results show that the ratio of ordered and disordered volume fractions in the mesodomain is exquisitely sensitive to the starting sucrose concentration.

INTRODUCTION

The freezing of unsaturated aqueous solutions of sugars at rates of $<10^3$ Kelvin per second (K/s) produces a heterogeneous state, in which a concentrated liquid volume fraction of the solute sugar is excluded from growing water-ice crystalline domains.¹ Electron microscopy (EM) studies show that this mesoscopic domain, or “mesodomain,” assumes the form of an interstitial network at the boundaries of the ice-crystalline (or grain) domains.^{1–3} The phase

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Author Contributions

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Supporting Information

Dependence of spin lattice relaxation rate on TEMPOL concentration in low temperature, homogeneous 60% (w/v) sucrose glass. This material is available free of charge via the Internet at <http://pubs.acs.org>.

behavior of the sucrose-water system upon freezing, which has been studied for over five decades,⁴ is described by the temperature-composition (T - c) state diagram in Figure 1. The T - c diagram is derived principally from calorimetric measurements.⁵ Figure 1 shows that, with increasing sucrose concentration, the liquidus curve (coexistence of water-ice crystals and fluid sucrose-water) passes through a theoretical eutectic point, T_e , where sucrose crystallization is kinetically restricted by the high viscosity of the sucrose-water mixture. The liquidus curve terminates at the glass transition temperature, T_g' , which corresponds to the value of $80 \pm 5\%$ (w/w) [$120 \pm 8\%$ (w/v)] sucrose.⁶ The enduring interest in the description of the physical properties of sugar-water solutions has been driven by the central role of sugars in food and biological sample preservation applications in industry and medicine.⁷⁻⁹ The detailed, microscopic characterization of sugar modulation of solvent-protein structural and dynamical coupling is also critical for the fundamental understanding of protein function.¹⁰⁻¹²

During “slow” ($<10^3$ K/s) cooling of an unsaturated sucrose-water solution, a trajectory to higher sucrose concentration along the “equilibrium” liquidus curve of Figure 1 is effectively reproduced within the mesodomain, as water removal from the mesodomain volume by water-ice crystal growth is driven by the lowering T . As T continues to decrease, more water-ice forms, the mesodomain volume decreases further, the sucrose concentration in the mesodomain increases, and the mesodomain eventually forms a glass, at $T_g'' < T_g'$. The position of the effective liquidus curve under finite cooling rates, and the value of the effective glass transition temperature, T_g'' , depend on the cooling rate and sample thermal history, as depicted by the dotted liquidus curve in Figure 1. At the slower cooling rate limit (approximately, $<10^2$ K/s), the sucrose in the mesodomain achieves the “maximally freeze-concentrated” value of $80 \pm 5\%$ (w/w) [$120 \pm 8\%$ (w/v)] sucrose,⁶ and the mesodomain forms a glass, with $T_g'' \approx 241$ K.¹ In contrast, a homogeneous glassy state over the entire sample volume is created by cooling of concentrated or super-saturated sugar solutions (Figure 1). For the cooling rates of ~ 10 K/s or lower, that are used in the studies reported here, the heterogeneous frozen state is formed at concentrations of $\sim 50\%$ (w/v), and the homogeneous glass is formed for concentrations of $>50\%$ (w/v) sugar. At ultra fast cooling rates ($>10^4$ K/s), glass formation supersedes homogeneous and inhomogeneous nucleation. Ultra-fast cooling studies show that pure water vitrifies at 138 K.¹³

TEMPOL, a relatively small (effective diameter, approximately 7 Å), paramagnetic ($S=1/2$) nitroxide molecule, is also excluded from the water-ice crystalline domains upon cooling of aqueous solutions. Therefore, TEMPOL is a localized, in situ electron paramagnetic resonance (EPR) spin probe of the mesodomain.¹⁴ TEMPOL has been used to characterize the dynamics of vitreous pure water^{14, 15} and aqueous glycerol solutions¹⁴ at low temperatures. These studies used continuous-wave (CW) EPR spectroscopy to observe motional (tumbling) effects on the TEMPOL line shape, from T variation-induced dynamical transitions in the surrounding solvent. This led to a calibrated T_g for pure water of 136 K,¹⁵ in agreement with the reported value obtained by extrapolation of calorimetric data.¹³ Calibrated T_g' values for the mesodomain in the heterogeneous frozen glycerol-water system at different glycerol concentrations were also determined.¹⁴ These studies suggest that application of high-resolution EPR spectroscopic techniques will reveal detailed microscopic information about sugar-water mesodomain structure.

Here, we apply multiple CW- and pulsed-EPR approaches to samples containing a constant, trace (0.2 mM, 0.0034%) concentration of TEMPOL spin probe, to characterization of microscopic parameters of the sucrose-water mesodomain, such as solute density and volume, in both heterogeneous liquid and solid states, over a wide range of added sucrose concentrations [0 – 75% (w/v)]. The following approaches were used to probe the solute concentration, volume, and microscopic structure of the mesodomain: (a) Continuous-wave

EPR spectroscopy of TEMPOL mobility (tumbling) was used to determine the dynamical transition (related to the effective glass transition temperature, T_g') in the mesodomain over 200-270 K. (b) Electron spin echo envelope modulation (ESEEM) spectroscopy at 6 K was used to detect the hyperfine interaction of TEMPOL with ^2H -labeled sucrose, as a probe of the relative volume of the mesodomain. (c) Electron spin echo (ESE) –detected longitudinal, or spin-lattice relaxation times (T_1) at 6 K were used to determine the concentration of TEMPOL in the mesodomain, and therefore, the volume fraction of the mesophase. (d) The ESE phase memory time (T_M ; correlated with the transverse relaxation time, T_2) at 6 K was also used as an indicator of the relative concentration of TEMPOL in the mesodomain. Integrated interpretation of the results leads to a novel microscopic model of a heterogeneous mesodomain structure, in which maximally freeze-concentrated sucrose¹ forms an ordered sucrose hydrate phase, that excludes solutes, and a disordered, amorphous sucrose-water glass phase. The volume fractions of the ordered and disordered phases are exquisitely sensitive to the initial, added sucrose concentration.

EXPERIMENTAL

Sample preparation

Sucrose (99.5%, Sigma-Aldrich, St. Louis, MO) and deuterated sucrose ($[\text{6,6'}\text{-}^2\text{H}_2]^{\text{fru}}$) sucrose (Omicron Biochemicals, Inc., South Bend, IN) were used without further purification. Water was purified to a specific impedance of 13.6 M Ω -cm (Nanopure, Siemens). Samples with different concentrations of sucrose [1-75% (w/v)] were prepared by mixing natural abundance or deuterated sucrose and water. The final concentration of TEMPOL (4-Hydroxy-TEMPO, Sigma-Aldrich, St. Louis, MO) spin probe in all samples was 0.2 mM [0.0034% (w/v)]. All samples were carefully degassed by freeze-thaw cycles before loading into standard quartz 4 mm EPR tubes (Wilmad-LabGlass, Buena, NJ). Samples were first frozen in a 252 K freezer and maintained at this temperature for 12 hours. Samples were subsequently transferred to liquid nitrogen (LN_2) for storage.

CW-EPR spectroscopy

CW-EPR experiments were performed at X-band on a Bruker Biospin ElexSys E500 system. An Oxford Instruments cryostat with continuous heat-exchanged N_2 flow and Bruker ER4131VT temperature controller were used to stabilize temperatures from 160 to 270 K. A microwave frequency of 9.41 GHz and power of 0.2 mW were used, except for samples with 60%, 75% (w/v) sucrose content, which required a lower power of 0.02 mW.

Simulations of the CW-EPR spectra were performed by using the program, EasySpin.¹⁶ Random rotational motion (tumbling) of the spin probe was assumed, and the corresponding correlation time, τ_c , was an adjustable parameter. A common range of parameters was used in the simulation of each spectrum at the different T values, as follows: $g_{xy}=2.009\text{--}2.010$, $g_z=2.004\text{--}2.005$; $A_{xy}=6.4\text{--}7.5$ Gauss, $A_z=36\text{--}39$ Gauss. The spectra were simulated by using a single component, with the exception of the spectra, that correspond to T_i , which required two components. For these two-component spectra, the weighted average τ_c value is plotted in Figure 3. The spectrum in Figure 2b, for 255 K is representative (normalized proportions, component 1: 0.6, component 2: 0.4; $\tau_{c1}=10$ ns, $\tau_{c2}=2.6$ ns).

Spin-lattice relaxation time measurement from dependence of ESE amplitude on pulse repetition rate

All electron spin echo (ESE) experiments were performed on a home-built X-band pulsed EPR spectrometer at 6 K. The two-pulse sequence, ($P_1\text{—}\tau\text{—}P_2\text{—}\tau\text{—}\text{echo}$), was used to generate the ESE for measurements of the longitudinal, or spin lattice, relaxation time, T_1 , by using the dependence of the ESE amplitude on pulse repetition rate.^{17, 18} A microwave

frequency of 8.752 GHz, magnetic field of 3115 G and τ value of 504 ns were used. Amplitudes of the ESE were recorded as a function of the pulse sequence repetition rate, from 1 to 1000 Hz. Dwell times between the pulse sequences were adjusted to ensure that equilibration of amplitudes was reached at all repetition rates. The dependence of the ESE amplitude on the pulse sequence repetition period is given by the following expression:^{17, 18}

$$V(t_{rep}) = V_0 \left[1 - \exp\left(-\frac{t_{rep}}{T_1}\right) \right] \quad \text{Eq. 2}$$

where V is the ESE amplitude, t_{rep} is the repetition period and V_0 is the maximum ESE amplitude as $t_{rep} \rightarrow \infty$. As the repetition period decreases (repetition rate increases), the spin system thermally equilibrates less between pulse sequences (saturates), and the ESE amplitude decreases.

Phase memory time measurement by using two-pulse ESEEM

The pulse sequence ($P_1 - \tau - P_2 - \tau - \text{echo}$), with $P_1 = P_2$,¹⁹ was used in the two-pulse ESEEM experiments on samples with varying percentages of sucrose. A microwave frequency of 8.752 GHz, magnetic field of 3115 G, and a repetition rate of 10 Hz were used, and $T = 6$ K. The baseline decay of the two-pulse ESEEM waveform is characterized by a stretched exponential function,¹⁸ and the following form was fitted to the decay data to obtain the phase-memory time, T_M , and exponent, n :

$$V(2\tau) = V(0) \exp\left[\left(-\frac{2\tau}{T_M}\right)^n\right] \quad \text{Eq. 3}$$

where $V(2\tau)$ is the ESE amplitude at a time, 2τ , after P_1 , and $V(0)$ is the ESE amplitude at $\tau = 0$.

Three-pulse ESEEM spectroscopy

The standard pulse sequence ($P_1 - \tau - P_2 - T - P_3 - \tau - \text{echo}$),¹⁸ with $P_1 = \frac{\pi}{2}$, was used in three-pulse ESEEM experiments. A microwave frequency of 8.752 GHz, magnetic field of 3115 G, $\tau = 226$ ns, and a pulse sequence repetition rate of 100 Hz were used, and $T = 6$ K. The selected τ of 226 ns suppresses modulation from solvent (matrix) ^1H and enhances modulation from weakly-coupled ^2H .

Simulations of ESEEM waveforms were based on the theory of Mims,²⁰ and were performed by using OPTESIM,²¹ with the assumption of a single ^2H coupled to the electron with adjustable hyperfine coupling parameters. The simulations indicate that $A_{\text{iso}} = 0$. The point dipole approximation, and a random orientation of electron and nuclear dipoles, were assumed, which leads to r_{en} as an adjustable parameter. The nuclear quadrupole coupling constant, e^2qQ/h , and electron field gradient asymmetry parameter, η , were fixed to 0.2 MHz and 0.1, respectively.²² In this study, we calculated the EMD values at the second trough position of the ESEEM waveform, as described in Results.

RESULTS

Mesodomain mobility transition of the TEMPOL paramagnetic probe from EPR lineshape analysis

EPR spectra for 0.2 mM TEMPOL in pure water and in 10 % (w/v) sucrose solution at different temperatures from the range, 180-270 K, are presented in Figure 2. The characteristic TEMPOL spectrum arises from interaction of the unpaired electron spin with the nitroxide ^{14}N nucleus (nuclear spin, $I = 1$), which produces three dominant spectral

features, that correspond to hyperfine coupling in the $m_I(0, \pm 1)$ states. The EPR spectrum of TEMPOL is sensitive to the probe's mobility (tumbling) on time scales of approximately $10^{-10} - 10^{-7}$ s.²³ The representative series of spectra in Figure 2 show that the line widths of the hyperfine features progressively narrow with increasing temperature, as increased solvent mobility²⁴ allows random rotational diffusive motion of the TEMPOL on shorter time scales, which enhances motional averaging of the anisotropic hyperfine contributions to the lineshape.²³ The EPR spectra for 180 K in Figure 2 show the rigid limit, powder pattern lineshape, which has an overall line width of $2A_{zz}=76$ Gauss=210 MHz, where A_{zz} is the z -component of the anisotropic hyperfine tensor. As the anisotropic contributions are averaged to a value of zero, the overall line width approaches $2A_{iso}$, where A_{iso} (17 Gauss) is the orientation-independent electron-¹⁴N isotropic hyperfine coupling constant. Representative simulations of the EPR spectra are also shown in Figure 2. The EPR simulations were performed, under the assumption of random rotational motion of TEMPOL, to obtain the correlation time, τ_c for the motion.

Values of τ_c for TEMPOL motion are plotted in Figure 3 as a function of temperature, for pure water and different sucrose compositions. The T -dependences of τ_c in Figure 3 display three common regimes: (a) At relatively low T , the upper-limit value of τ_c corresponds to the immobilized spin probe, and τ_c is not accurately specified by the tumbling model. The average value of τ_c from simulations of the rigid limit spectra are shown in Figure 3. (b) The sharp decrease in τ_c corresponds to the transition to the tumbling regime, and TEMPOL rotational motion on the time scale of the inverse linewidth ($2A_{zz}^{-1}, \sim 10^{-8}$ s). The temperature at the termination of the transition is defined as T_t . (c) At $T > T_t$, τ_c decreases further, with a less sensitive dependence on T , owing to the thermally activated rotational motion of the spin probe. The value of T_t is $\ll T_m$ for bulk water, which indicates that TEMPOL occupies a mesodomain in pure water and in sucrose-water binary mixture, as reported for other binary solvent systems.^{14, 15} Figure 3 shows that $T_t=205$ K for TEMPOL in pure water. Figure 3 also shows the dependence for added 1, 3, 5, 10, 30 and 60 % (w/v) sucrose. Addition of 1% (w/v) sucrose leads to a large, 35 K shift in T_t to 240 K. Increments in T_t decrease with further increases in sucrose concentration, and converge to a constant value of $T_t=255 \pm 5$ K for 3% (w/v) sucrose. The TEMPOL line shape dependence on temperature at different sucrose concentrations reveals two dominant types of mesophase environment. The mesodomain environment in pure water is characterized by a relatively low- T threshold for TEMPOL motion ($T_t=205$ K). The second type of environment is common among the sucrose-water mesophases in samples of 3–50 % sucrose (w/v), and is therefore relatively independent of the amount of added sucrose. This environment is characterized by a relatively high- T threshold for TEMPOL motion ($T_t=255 \pm 5$ K).

Relative sucrose concentration in the mesodomain from electron spin echo envelope modulation (ESEEM) spectroscopy

ESEEM is a pulsed-EPR experiment that characterizes relatively weak hyperfine coupling of the unpaired electron spin with magnetic nuclei, in the low-temperature solid state (here, 6 K).²⁰ In ESEEM, the amplitude of the electron spin echo (ESE), is recorded as a function of time interval between the microwave pulses, that are used to manipulate the electron spins to form the ESE. In the one-dimensional, 3-pulse sequence used here, ($P_1 - \tau - P_2 - T - P_3 - \tau$ — ESE; P_i , microwave pulse, $P_i = \pi/2$; τ , dephasing time; T , waiting time),¹⁸ the ESE dependence on varied T leads to the ESEEM waveform. The ESEEM waveform is modulated at the precession periods of nuclear spins, that are hyperfine-coupled to the unpaired electron spin, and the amplitude of the modulation relative to the unmodulated amplitude of the ESE, or envelope modulation depth (EMD), reports on the nature of the coupling.¹⁸ Figure 4a shows representative 3-pulse ESEEM from coupling of the TEMPOL

electron spin with ^2H nuclei ($I=1$) on $[6,6'\text{-}^2\text{H}_2^{\text{fru}}]\text{-sucrose}$. The modulation period of 480 ns corresponds to the inverse of the 2.1 MHz (0.75 Gauss) Larmor frequency of “free” ^2H at the magnetic field of 3115 Gauss. Simulations of the ^2H ESEEM were performed by using OPTESIM.²¹ The simulations indicate that $A_{\text{iso}}=0$ for the ^2H coupling, and thus, the detected ^2H are not in direct, bonded contact with TEMPOL. In this limit of weak, “matrix” hyperfine coupling, the EMD is proportional to the number of coupled nuclei, and to the electron nuclear separation distance, as r_{en}^{-3} . The EMD arises from dipolar hyperfine interactions of the unpaired electron spin with relatively distant ^2H nuclei on $[6,6'\text{-}^2\text{H}_2^{\text{fru}}]\text{-sucrose}$ in the environment around TEMPOL, and therefore, report on the local concentration of sucrose.

Figure 4b shows the EMD of samples as a function of concentration of $[6,6'\text{-}^2\text{H}_2^{\text{fru}}]\text{-sucrose}$, over a range of 1 – 75% (w/v) added sucrose. Values of the EMD (determined as defined in Figure 4a) are presented in Table 1. The ESEEM simulations indicate that a single coupled ^2H , with effective r_{en} values of $6 \pm 2 \text{ \AA}$, accounts for the dominant portion of the EMD, for 3 – 75% (w/v) added sucrose. The results show that, with the exception of 1% (w/v) sucrose, the EMD remains constant, to within the standard deviation, as a function of the added sucrose concentration. Therefore, the local concentration of sucrose around TEMPOL is constant, to within the standard deviation of the measurements, at added concentrations of sucrose, 1% (w/v).

The sensitivity of the ESEEM method to the concentration of ^2H -sucrose was assessed, by comparison with the EMD from a sample that contains 30% (w/v) $[6,6'\text{-}^2\text{H}_2^{\text{fru}}]\text{-sucrose}$ and 30% (w/v) natural abundance sucrose [total sucrose: 60% (w/v)]. At 60% (w/v) sucrose, the sample is a homogeneous glass, and this leads to the random distribution of the sucrose throughout the sample. Figure 4b shows that the EMD of the 1:1 ^2H -sucrose/ ^1H -sucrose sample is approximately half of the 60% (w/v) $^2\text{H}_2\text{-sucrose}$ sample. This demonstrates that the EMD measurement is capable of detecting significant changes in the local concentration of ^2H -sucrose.

Relative TEMPOL mesophase concentration from spin-lattice relaxation time in sucrose solutions

The longitudinal, or spin-lattice, relaxation time (T_1) of electron spin polarization decreases as the concentration of paramagnets increases.¹⁸ This is described by the Redfield theory,²⁵ which relates the spin-spin interactions to the distance-dependent electron-electron dipolar coupling, and analytical expressions for T_1 (and spin-spin relaxation time, T_2) have been derived.^{26, 27} Therefore, the value of T_1 in the samples is sensitive to the concentration of TEMPOL. The T_1 was determined from the dependence of the 2-pulse ESE amplitude on the pulse sequence repetition rate,¹⁷ at $T=6 \text{ K}$. A maximum 2-pulse ESE amplitude is achieved, when the spin system in the sample return, by spin-lattice relaxation, to the equilibrium magnetization, after each microwave pulse sequence ($P_1 - \tau - P_2 - \tau - \text{ESE}$; $P_1=P_2$).¹⁹ As the pulse sequence repetition rate increases, and becomes comparable to T_1^{-1} , the spin system does not relax fully to equilibrium between pulses, and the ESE amplitude declines. At pulse repetition rates $\gg T_1^{-1}$, the spin system is saturated, and the ESE amplitude is zero. The relation between the ESE amplitude and pulse repetition rate is given by Eq. 1.¹⁷ The samples contained a constant concentration of 0.2 mM TEMPOL and different amounts of added sucrose. Figure 5a shows the dependence of the ESE amplitude on pulse sequence repetition rate for samples with varying sucrose concentrations, and the fits of Eq. 1 to the data. As the concentration of added sucrose increases (right-to-left, in Figure 5a), the pulse repetition rate required to achieve saturation of the spin system (ESE amplitude equal to zero) decreases, indicating that T_1 increases. The exponential ESE decay dependence with a single value of T_1 provides a very good fit to the data, at each sucrose

concentration. This indicates that the TEMPOL occupies a single type of environment in the mesodomain, at each sucrose concentration.

Figure 5b shows the T_1 values for TEMPOL as a function of sucrose concentration. The T_1 values and fitting errors are collected in Table 1. The general trend of increasing T_1 (slower relaxation) of the spin system with increasing added sucrose concentration, at a constant amount of added TEMPOL, indicates that the concentration of TEMPOL in the mesodomains decreases, as the concentration of added sucrose increases. Thus, the volume of the mesophase increases with added sucrose. Three regimes of T_1 dependence on the added sucrose concentration, which are separated by clearly delineated transitions, are displayed in Figure 5b: 1-15%, 20-50%, and >50% (w/v).

Calibration of TEMPOL concentration with spin-lattice relaxation time in glassy 60% (w/v) sucrose solution

To quantify the TEMPOL concentration in the mesophase, an independent calibration measurement was performed in homogeneous glass samples at 60% (w/v) sucrose, in which the TEMPOL is randomly distributed. The T_1 was measured at 6 K as a function of TEMPOL concentration, from 0.12-100 mM. The range of corresponding T_1 values was approximately 1 – 300 s. The dependence of TEMPOL concentration on T_1 , plotted as the logarithm of TEMPOL concentration as a function of logarithm of inverse T_1 (T_1^{-1} ; rate of longitudinal spin relaxation), is shown in Figure S1 (Supporting Information). As described in Experimental, the log-log plot best portrays the form of the empirical cubic polynomial function that is used to fit the data. The function provides the concentration of TEMPOL directly from the measured T_1 values. The application of the fit of the relation in Figure S1 to estimate the effective concentration of TEMPOL in the mesodomain is considered in the Discussion.

Relative TEMPOL mesodomain concentration from phase memory relaxation time in aqueous sucrose solutions

The decay of the ESE amplitude as a function of the dephasing time, τ , is characterized by the stretched exponential function, Eq. 2,¹⁸ with phase memory time constant, T_M . The value of T_M is significantly shorter than T_1 , because transverse relaxation does not require energy exchange with the environment.^{18, 23} The T_M value for TEMPOL, in the frozen sucrose samples at 6 K, is dominated by the relaxation of the transverse components of the electron spin polarization, owing to the dipolar coupling among the electron spins.²³ Therefore, T_M is dependent on the distance between electron spins, and is thus sensitive to the local concentration of TEMPOL. Figure 6a shows the 2-pulse ESE amplitude as a function of τ , at representative added sucrose concentrations. The fitted values of T_M are presented in Table 1. Figure 6b shows that the dependence of T_M on sucrose concentration follows a similar trend as for the spin-lattice relaxation time T_1 : T_M increases as the amount of added sucrose increases. Thus, the T_M dependence indicates that there is a general increase in the volume of the mesodomain (corresponding to an increase in the average electron-electron separation distance), with added sucrose. Figure 6b also shows that, as observed for the T_1 , there are three dominant regimes of T_M dependence on added sucrose concentration, as follows: 1-15, 20-50, and >50 % (w/v).

DISCUSSION

Identification of the mesodomain and properties in pure water and 1% (w/v) sucrose solution

The T_f value of 205 K for pure water is significantly less than the melting temperature, $T_m=273$ K, for the bulk solvent, but significantly greater than the T_g value of 136 K¹³⁸

K.^{13, 14} This provides direct evidence that TEMPOL is excluded from the water-ice crystalline domains, and that it is present in the interstitial mesodomain. Figure 3 shows that addition of 1% (w/v) sucrose leads to a shift in T_i to 240 K, relative to pure water. This shift is less than the shift to 255 ± 5 K for >1% (w/v) sucrose solutions. Figure 4b shows an EMD for 1% (w/v) sucrose that is approximately one-half of the EMD for >1% (w/v) sucrose. The deviations in the spin probe mobility and ^2H -ESEEM for 1% (w/v) sucrose, relative to the uniform T_i and EMD values for higher sucrose concentrations, may result from a partial breakdown of the free probe assumption²⁸ in the mesodomain, at very low starting sucrose concentrations.

Relative volume of the mesodomain in unsaturated sucrose-water solutions

Figure 3 shows that sucrose concentrations of >1% (w/v) lead to T_i values that are approximately uniform (255 ± 5 K). The motional averaging of the anisotropy in the EPR lineshape is related to the tumbling motion of the TEMPOL probe. The time scale of the isotropic probe tumbling is approximated by the Stoke's law rotational correlation time, $\tau_{c,S}$:²³

$$\tau_{c,S} = \frac{4\pi\eta a^3}{3k_B T} \quad \text{Eq. 1}$$

where η is the solvent viscosity, a is the probe radius, and k_B is the Boltzmann constant. As shown in Eq. 1, τ_c is directly proportional to η . The comparable T_i values for the sucrose-water samples thus indicate that the mesodomain viscosity is comparable at each temperature, for >1% (w/v) sucrose. The viscosity of sucrose-water mixtures is dependent on the sucrose concentration.^{29, 30} Therefore, the results indicate that the sucrose concentration in the mesophase is approximately constant, for >1% (w/v) sucrose. The comparable EMD values for the TEMPOL-[6,6'- $^2\text{H}_2$ ^{fru}]-sucrose ESEEM in Figure 4b and Table 1, also show that the mesodomains formed by added concentrations of sucrose of >1% (w/v) have a comparable freeze-quenched sucrose concentration in the mesodomain. This result is consistent with the standard assumption that the composition of the melt state of a system at a relatively high T (here, 200-270 K) is preserved in the "quench" state at lower T (here, at 6 K, after quenching).

Overall, the spin probe mobility and ^2H -ESEEM measurements are consistent with a maximally freeze-concentrated value for the sucrose concentration in the mesodomain,¹ for each concentration of added sucrose at >1% (w/v). The maximal freeze-concentrated concentration has been proposed¹ to correspond to T_g' , as defined in Figure 1. An estimate of the concentration will be provided, below.

Spin probe concentration in the mesodomain

The general trend in the values of T_i at 6 K, obtained from the results presented in Figure 5b, and summarized in Table 1, indicates that the volume of the mesodomain increases with increasing added sucrose concentration, which is consistent with a maximal freeze-concentrated sucrose-water mesodomain. The T_i -sucrose concentration calibration curve in Figure S1 allows calculation of the effective concentration of TEMPOL in the mesodomain. Figure 7 shows the calibrated TEMPOL mesodomain concentration as a function of added sucrose concentration. The dashed line in Figure 7 is the predicted TEMPOL concentration,¹ corresponding to the assumption of $80 \pm 5\%$ (w/w) [$120 \pm 8\%$ (w/v)] sucrose,⁶ present as an amorphous glass. The homogeneous glass-forming 60 and 75% (w/v) super-saturated sucrose samples obey the relation, to within the standard deviation of the measurements. However, the unsaturated solution samples exhibit effective TEMPOL concentrations that are 5- to 10-fold higher than the prediction. Further, the dependence is punctuated by

transitions at 15-20% and 50-60% (w/v) added sucrose. The T_M values in Figure 6b also show a three-stage, two-transition dependence on added sucrose concentration. Thus, both the T_1 and T_M results suggest that the mesodomain is not homogeneous.

Heterogeneous structure of the mesodomains formed from unsaturated sucrose-water solutions

The deviation of the TEMPOL concentration from the value predicted for maximally freeze-concentrated 80% (w/w) sucrose,¹ which is illustrated in Figure 1, is attributed to the formation of crystalline sucrose hydrate structures in the mesodomain. We propose that the mesodomain contains a sucrose hydrate fraction that excludes TEMPOL, and an amorphous solid sucrose-water fraction, in which the TEMPOL resides. Figure 8 shows the ratio of the volume fractions of sucrose hydrate (ordered) and amorphous solid sucrose-water (disordered). The volume fractions are calculated from the data in Figure 7.

Support for the proposed model of mesodomain heterogeneity comes from evidence for sucrose hydrates in aqueous sucrose solution measurements on non-mesodomain phases, as follows: (a) The species, sucrose hemipentahydrate ($C_{12}H_{22}O_{11} \cdot 2.5 H_2O$) and sucrose hemiheptahydrate ($C_{12}H_{22}O_{11} \cdot 3.5 H_2O$), form upon extended exposure of aqueous sucrose samples to a temperature between the T_m and T_g of water.⁴ (b) DSC measurements indicate partitioning of the temperature-composition state diagram into sub-regions, according to the existence of different sucrose hydrates.³¹ (c) The possible influence of sucrose-solvent and sucrose-sucrose hydrogen bonding interactions on composition and phase segregation in fluid aqueous sucrose solutions is implicated by Raman³² and fourier transform infrared (FTIR)³³ spectroscopic and X-ray³⁴ scattering studies. An ordered sucrose fraction was also identified in the mesophase.³⁴

Figure 8 shows that the ratio of sucrose hydrate and amorphous sucrose-water volume fractions is segregated into three distinct regions, as follows: (a) At low initial concentrations of sucrose of <20% (w/v), sucrose hydrate formation is facilitated by the increasing sucrose concentration. (b) At intermediate starting sucrose concentrations of 20 % (w/v) – 50, about 80% of the sucrose forms hydrate structure, and this ratio is constant across this range. (c) At high starting sucrose concentration of >50% (w/v), all sucrose forms an amorphous aqueous glass, which is homogeneous over the sample volume. The graded decrease in sucrose hydrate formation as the starting concentration of sucrose increases up to 50% (w/v) may be caused by an increasing effective viscosity of the mesodomain, which suppresses hydrate nucleation and propagation. Unsaturated aqueous sucrose solutions show discontinuities in the following physical properties at 47% (w/v) sucrose solution: (a) viscosity³⁵, (b) sucrose C-O-C bending frequency probed by Raman spectroscopy,³² and (c) water mobility probed by 1H and ^{17}O nuclear magnetic resonance (NMR) spectroscopy.^{36, 37} The diffusion of water and sucrose decouples above 50% (w/w) sucrose.³⁸ The transition in Figure 8 at 50-60% (w/v) added sucrose therefore appears to be associated with the transition from heterogeneous frozen solution to the homogeneous glass.

CONCLUSIONS

Unsaturated aqueous sucrose solutions form a heterogeneous structure upon freezing, in which a sucrose-water mesoscopic domain (mesodomain) exists at the boundaries of water-ice crystalline domains. The pre-freezing concentration range of 0-75% (w/v) sucrose spans pure water, unsaturated and supersaturated sucrose solution conditions. Spectroscopic and relaxation techniques of CW- and pulsed-EPR were applied by using the mesodomain-localized TEMPOL spin probe, to characterize the environment and determine the absolute volume of the mesodomain over the range of added sucrose concentrations. The different EPR techniques lead to a set of consistent results. The measured mesodomain volume is less

than the predicted maximally freeze-concentrated volume, with transitions in the volume-composition dependence at two starting concentrations, 15-20% and 50% (w/v) sucrose. Ordered sucrose hydrate and disordered amorphous sucrose-water glass phases are proposed to compose a heterogeneous mesodomain. The results provide unprecedented resolution of the mesodomain structure in frozen aqueous sucrose solutions, and show that the ratio of ordered and disordered volume fractions depends sensitively on the starting sucrose concentration. The fundamental understanding of microscopic structure and order/disorder transitions in the mesodomain is critical for characterization of the structural and dynamical contributions of solvent-protein interactions to protein function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Equilibrium temperature-composition phase diagram for water-sucrose mixtures, and overlaid cooling-rate dependences of the relations. Representative cooling trajectories from a room temperature solution of 30% sucrose (w/v) are depicted. When the temperature drops to the freezing line, water crystallizes and the sucrose concentration increases around the water-crystalline domains. As temperature continues to drop, the trajectory follows the equilibrium liquidus curve for infinitely slow cooling (solid arrows) through the eutectic (T_e), and reaches a maximally freeze-concentrated state at the glass transition (T_g'). For finite cooling rates of $<10^3$ K/s, the trajectory follows an apparent liquidus curve (dashed line, arrows) and forms a glass at a lower temperature, T_g'' . At T_g' (or T_g'') the sucrose is maximally freeze-concentrated in the mesodomain, at the boundaries of the ice crystalline regions.

Figure 2. CW-EPR spectra of TEMPOL spin probe in pure water and aqueous sucrose solutions, at different temperatures, and overlaid EPR simulations (dashed, red line). (a) 0% sucrose (pure water). (b) 10% (w/v) sucrose. The concentration of TEMPOL is 0.2 mM (0.0034%).

Figure 3.

Temperature dependence of the rotational correlation time of the TEMPOL spin probe in pure water and in representative aqueous sucrose solutions. Values of τ_c were derived from simulations of the CW-EPR spectra. The average value of τ_c from the rigid limit simulations (at 5 K increments from 160 K to the mobility transition temperature) is shown (○). Symbols: (◁) 0%, (▷) 1%, (▽) 3%, (+) 5%, (△) 10%, (×) 30%, (◇) 60% (w/v) sucrose.

Figure 4.

Three-pulse ESEEM from the TEMPOL spin probe in aqueous sucrose solutions at different concentrations of [6,6'- $^2\text{H}_2^{\text{fru}}$] sucrose. (a) Three-pulse ESEEM waveforms (black), and overlaid ESEEM simulations (red). (Top) 50% (w/v) sucrose. (Middle) 20% (w/v) sucrose. (Bottom) 5% (w/v) sucrose. The EMD is calculated from the ESE amplitude at the second trough and an interpolation of the first and second peaks, as indicated by the dashed line. Vertical scale bar in bottom panel represents 5% of the unmodulated ESE amplitude. (b) Dependence of the three-pulse ESEEM envelope modulation depth (EMD) on [6,6'- $^2\text{H}_2^{\text{fru}}$] sucrose concentration (black circles). Control sample with 30% (w/v) [6,6'- $^2\text{H}_2^{\text{fru}}$] sucrose and 30% (w/v) natural abundance (^1H) sucrose [total, 60% (w/v) sucrose] (red square).

Figure 5.

Spin-lattice relaxation of TEMPOL in pure water and in aqueous sucrose solutions at different sucrose concentrations. (a) Pulse repetition rate dependence of the normalized two-pulse ESE amplitude of TEMPOL in pure water and in aqueous sucrose solutions with different sucrose concentrations, [% (w/v)]: (+) 0, (○) 1, (*) 3, (●) 5, (×) 10, (◐) 15, (◑) 20, (▽) 25, (△) 30, (▷) 40, (◐) 50, (●) 60, (■) 75. Solid curves represent fits to Eq. 2. (b) Dependence of the spin-lattice relaxation time, T_1 , of the TEMPOL spin probe on sucrose concentration. The T_1 values were obtained by fits of Eq. 2 to the curves in (a).

Figure 6.

Phase memory relaxation of TEMPOL in pure water and in aqueous sucrose solutions at different sucrose concentrations. (a) Two-pulse ESEEM waveforms for the TEMPOL spin probe for pure water and different aqueous sucrose solutions [% (w/v)], and overlaid best-fit stretched exponential decay function (Eq 3.; dashed line). (b) Dependence of the phase memory relaxation time, T_M , of the TEMPOL spin probe on sucrose concentrations. The T_M values were obtained by fits of Eq. 3 to the curves in (a).

Figure 7.

Dependence of the effective TEMPOL concentration on sucrose concentration in different aqueous sucrose solutions. The dashed line is the predicted TEMPOL concentration, under the assumption that all sucrose forms $80 \pm 5\%$ (w/w) [$120 \pm 8\%$ (w/v)] sucrose maximally freeze-concentrated amorphous aqueous-sucrose glass.⁶

Figure 8.

Dependence of the ratio of volume fractions of the ordered (sucrose hydrate) and disordered (amorphous aqueous-sucrose) phases of the heterogeneous mesodomain on sucrose concentration in different aqueous sucrose solutions.

Table 1

Electron spin echo envelope modulation, spin-lattice relaxation and phase memory relaxation parameters for the TEMPOL spin probe in pure water and in aqueous sucrose solutions at different concentrations of added sucrose.

Sucrose Concentration [% (w/v)]	EMD	T_1 (ms)	T_M (μ s)	n
0	–	2.1 $\pm$ 0.4	0.31 $\pm$ 0.03	3.2 $\pm$ 1.1
1	0.013 $\pm$ 0.007	5.0 $\pm$ 0.9	0.30 $\pm$ 0.07	1.5 $\pm$ 0.4
3	0.027 $\pm$ 0.002	6.4 $\pm$ 0.6	0.38 $\pm$ 0.10	1.1 $\pm$ 0.3
5	0.038 $\pm$ 0.005	25 $\pm$ 2	0.70 $\pm$ 0.09	1.2 $\pm$ 0.2
10	0.029 $\pm$ 0.004	50 $\pm$ 3	0.88 $\pm$ 0.11	1.1 $\pm$ 0.2
15	0.037 $\pm$ 0.002	69 $\pm$ 5	0.99 $\pm$ 0.12	1.2 $\pm$ 0.1
20	0.041 $\pm$ 0.004	270 $\pm$ 20	1.76 $\pm$ 0.08	1.7 $\pm$ 0.2
25	0.042 $\pm$ 0.006	440 $\pm$ 20	1.97 $\pm$ 0.06	1.8 $\pm$ 0.2
30	0.037 $\pm$ 0.006	380 $\pm$ 20	2.09 $\pm$ 0.05	2.0 $\pm$ 0.1
40	0.030 $\pm$ 0.006	490 $\pm$ 40	2.08 $\pm$ 0.06	1.9 $\pm$ 0.2
50	0.034 $\pm$ 0.006	510 $\pm$ 40	2.13 $\pm$ 0.09	1.9 $\pm$ 0.2
60	0.031 $\pm$ 0.006	810 $\pm$ 110	2.20 $\pm$ 0.08	2.6 $\pm$ 0.2
75	0.030 $\pm$ 0.006	1060 $\pm$ 150	2.39 $\pm$ 0.08	2.6 $\pm$ 0.2