Exploring the conformational energy landscape of glassy disaccharides by cross polarization magic angle spinning $^{13}$C nuclear magnetic resonance and numerical simulations. II. Enhanced molecular flexibility in amorphous trehalose

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This paper uses chemical shift surfaces to simulate experimental $^{13}$C cross polarization magic angle spinning spectra for amorphous solid state disaccharides, paying particular attention to the glycosidic linkage atoms in trehalose, sucrose, and lactose. The combination of molecular mechanics with density functional theory/gauge invariant atomic orbital ab initio methods provides reliable structural information on the conformational distribution in the glass. The results are interpreted in terms of an enhanced flexibility that trehalose possesses in the amorphous solid state, at least on the time scale of $^{13}$C nuclear magnetic resonance measurements. Implications of these findings for the fragility of trehalose glass and bioprotectant action are discussed. © 2007 American Institute of Physics. [DOI: 10.1063/1.2409935]

I. INTRODUCTION

The understanding of the local structure of inorganic amorphous states, such as metallic and oxide glasses, has made giant steps forward in recent decades, on both the experimental and theoretical fronts. Due to the rather simple elementary unit in these systems, the details of the local structure can often be very accurately described using a coordination shell image. Thus, most information on the structures of these simple glasses is in the form of radial distribution functions or their reciprocal counterparts (structure factors) that are accessible through neutron or x-ray scattering experiments. However, that approach rapidly becomes inadequate when molecular asymmetry or specific and directional interactions such as hydrogen bonds are present. Building a correct structural model of disordered, hydrogen-bonded networks is still a fascinating challenge. For example, see Ref. 2 for one of the innumerable studies on water. More complex hydrogen-bonded glasses, consisting of proteins, polyalcohols, or saccharides, are at the frontiers of the biophysical, food, pharmaceutical, or materials sciences. The rich complexity of carbohydrate systems, compared to the model inorganic glasses mentioned above, originates from their large number of intermolecular degrees of freedom. These coordinates necessarily affect the dynamic properties of the compound near its glass transition, often conferring a fragile character to the glass-forming molecule and also making the description of the glass structure a very complicated task. Only recently, local investigation techniques such as nuclear magnetic resonance (NMR) and computational molecular dynamic simulations have become useful.

Despite the large number of degrees of freedom arising from the numerous primary and secondary alcohol groups, a relatively small number of geometrical parameters are thought to provide a reasonable picture of disaccharide energy landscapes. Among disaccharides, trehalose has received considerable interest, mainly because of its biopreservation properties. At the molecular level, the mechanisms of preservation in the face of freezing or dehydration remain an open question. Trehalose has also been proposed as a model hydrogen bond network that is well described by the axiomatic theory of ideally glassy networks developed by Phillips. Molecular modeling by empirical force fields has been used often to explore the conformational properties of carbohydrates. One finding is that the resulting energy maps may have two or three potential energy wells that correspond to quite different but accessible conformations. Whether all of the wells are populated is still a matter of
The aim of the present work is to exploit the methodology assessed in the preceding paper\(^{14}\) for analyzing and comparing the conformational space that is actually accessible to three homologous sugars (trehalose, sucrose, and lactose) in their respective pure amorphous phases. The general method relies on the comparison between the preferred molecular geometries predicted with empirical force fields and the actual distribution of conformations that is inferred from\(^ {13}C\) solid state NMR on amorphous samples. The scientific background of the method and the simulation setup have been discussed in detail elsewhere.\(^ {11}\) Therefore, after a brief description of the experimental and computational conditions (Sec. II), the\(^ {13}C\) cross polarization magic angle spinning (CPMAS) NMR experimental results are presented. The results of the simulations are then illustrated and discussed in relation to the conformational flexibility of sugars and their relevance to glassy properties.

II. EXPERIMENTS AND METHODS

A. Samples

Anhydrous\(^ {\alpha,\alpha}\)-trehalose\((T_{\beta})\), stable anhydrous\(^ {\alpha}\)-lactose, and sucrose crystalline powders were purchased (Sigma-Aldrich) and used without further purification. The amorphous samples were obtained by ball-milling\(^ 1\) g of crystalline sugar during 30 h under dry nitrogen atmosphere in a planetary grinder Pulverisette 7 (Fritsch, Inc.). This milling time assured the complete loss of the crystalline form of trehalose, according to the absence of a melting signature in differential scanning calorimetry thermograms that also validated the total extent of decrystallization.\(^ {21}\) No yellowish color was found in the milled samples, assuring that no significant chemical degradation occurred.

B. NMR experiments

The\(^ {13}C\) CPMAS experiments were carried out at 100.6 MHz on a Bruker AV400 solid state NMR spectrometer. The linear amplitude modulation of the rf field during the contact pulse (typically 2 ms) and two pulse phase modulated heteronuclear decoupling during acquisition were employed. Recycle delays ranging between 200 and 450 s were used. The rotation speed was set to 5 kHz. A standard digital filter was used for acquisition, and the spectra were obtained by simple Fourier transform of the induction decay, without data apodization.

C. Molecular modeling

Hyperchem Pro 7 software (Hypercube, Inc.) was used for the molecular modeling. Conformations were optimized by relaxing all degrees of freedom except for the two dihedral angles\(\phi\) and\(\psi\), as defined in Table I for the three disaccharides. The Bio85 parameter set by Reiter III\(^ {22}\) for the CHARMM potential function was used. To take into account the mean field contributions of surrounding molecules in a condensed phase to the intramolecular interactions, an averaged dielectric constant\[\varepsilon_{\text{eff}}(r) = \varepsilon_0 \frac{\sigma(r)}{\sigma_{\text{calc}}(r)}\] was used, a standard procedure for screening Coulombic interactions. The 1–4 scale factors were equilibrated between Coulomb and van der Waals interactions.

D. Conformational energy maps

All calculations were started from the molecular coordinates obtained by the single crystal structure determination on anhydrous trehalose\((T_{\beta})\),\(^ {23}\) lactose monohydrate\((\alpha\text{-lactose})\),\(^ {24,25}\) and sucrose.\(^ {26}\) Each of these crystal structures was relaxed by molecular mechanics in order to find the lowest energy molecular conformation (LMC) in the Bio85 force field. Then, for each sugar a quasidiabatic Ramachandran map\(E(\phi, \psi)\) was calculated by classically mapping the whole\((\phi, \psi)\) space by 324 points separated by 20° steps.\(^ {6,20,27}\) For each\(\phi, \psi\) point in the map, the\(\phi\) and\(\psi\) values were set starting from the LMC coordinates, and then were restrained by setting a high value of dihedral angle spring constants. All other degrees of freedom were then relaxed using the Bio85 force field parameters.

E. Isotropic\(^ {13}C\) chemical shift maps

NMR chemical shift calculations were carried out for each studied disaccharide on the previous 324 relaxed conformations generated by molecular mechanics. For each conformation, the isotropic magnetic shielding of each carbon of the sugar was evaluated using the gauge invariant atomic orbital (GIAO) method, B3PW91 density functional theory, and the 3-21+\(G^*\) basis set, as implemented in the Gaussian 03 software (Gaussian, Inc.).\(^ {28}\) The 324 single point calculations resulted for each sugar in two magnetic shielding maps, that were converted into chemical shift maps\(\sigma(C_x, \phi, \psi)\) and\(\sigma(C_y, \phi, \psi)\), where\(C_x\) and\(C_y\) represent the two carbons involved in the glycosidic bond of the disaccharide. The conversion from rough magnetic shielding to a chemical shift comparable to the experiment was carried out by simple linear transformation\(\sigma = A\sigma_{\text{calc}} + B\), where\(A\) and\(B\) coefficients are given in Table II.\(^ {31}\)

<table>
<thead>
<tr>
<th>TABLE I. Dihedral angles (\phi) and (\psi) defining the glycosidic torsion angles of (\alpha, \alpha)-trehalose, (\alpha)-lactose, and sucrose.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\phi)</td>
</tr>
<tr>
<td>(\psi)</td>
</tr>
<tr>
<td>(\alpha)-trehalose</td>
</tr>
<tr>
<td>(\phi)</td>
</tr>
<tr>
<td>(\psi)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE II. Coefficients of the linear conversion (\sigma = A\sigma_{\text{calc}} + B) from calculated magnetic shielding (\sigma_{\text{calc}}) to chemical shift (\sigma).</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
</tr>
<tr>
<td>(\alpha, \alpha)-trehalose</td>
</tr>
<tr>
<td>(A)</td>
</tr>
<tr>
<td>(B)</td>
</tr>
</tbody>
</table>
F. Simulation of the CPMAS spectrum

The scientific background leading to the formulation of the simulated $S(\delta)$ CPMAS spectrum was presented and discussed in detail elsewhere.\textsuperscript{11} The numerical evaluation of $S(\delta)$ requires integration of constant chemical shift contours in the map $\sigma(C_\alpha, \phi, \psi)$, using Eq. (1).

$$S(\delta) = \int_{\sigma(C_\alpha, \phi, \psi)=\delta} \frac{\chi(\phi, \psi)}{\nabla \sigma(\phi, \psi)} d\phi d\psi. \quad (1)$$

In this equation, $\chi(\phi, \psi)$ represents the probability density of occupation of a given molecular conformation $(\phi, \psi)$.\textsuperscript{11} The $\chi(\phi, \psi)$ function carries all of the structural information that can, in principle, be extracted from the modeled NMR spectrum. The actual resolution of this map is limited in the present work to $20^\circ$ angular steps. In order to smoothen the integration contour, cubic spline interpolation was used. The simulated CPMAS spectrum $S(\delta)$ was then evaluated by sampling 1024 different $\delta$ values, each one corresponding to one numerical integration over an interpolated $\sigma(C_\alpha, \phi, \psi)=\delta$ contour, evaluated by the standard Romberg method. The gradient term in Eq. (1) was calculated by the finite difference approximation using $\Delta \phi=\Delta \psi=0.01^\circ$ angular steps.

The last step of data processing is the convolution of $S(\delta)$ with a Lorentzian function, in order to account for the homogeneous CPMAS linewidth. This was achieved by the inverse Fourier transform of $S(\delta)$ to a free induction decay $S(t)$, then multiplication of $S(t)$ by an exponential function, and back to the Fourier transform to $S(\delta)$. A typical line broadening imposed in this work was 1 ppm half width at half maximum.

III. RESULTS AND DISCUSSION

A. $^{13}$C CPMAS spectra

Figure 1 compares the CPMAS spectra of crystalline and amorphous forms of three anhydrous disaccharides, showing the resonances assigned to the glycosidic carbons. A broadening of all NMR signals is common to the three amorphous sugars. The distribution of isotropic chemical shifts reflects the underlying distributions of molecular conformations in the glass, which largely rely upon the difference in the glycosidic dihedral angles. It should be clear that a similar conformational distribution would exist in the liquid state (either pure or in solution); however, in this latter case the fast intramolecular conversion should give rise to a statistically averaged value (over time) of the chemical shift. For the glycosidic carbons, the observed chemical shift distributions spread over about 10 ppm for sucrose and lactose. For trehalose, it is highly asymmetric, tails downfield, and extends over more than 15 ppm. This extra range suggests that trehalose molecules experience enhanced flexibility in the glass in comparison with the other disaccharides, provided that the gradients in the chemical shift maps are similar.

Another remarkable point is that the NMR spectrum of the amorphous states does not completely envelope that of their respective crystalline states. In particular, the maxima of the chemical shift distributions of the glycosidic carbons of lactose and sucrose do not coincide with the NMR lines of the crystal forms. For trehalose, one of the $C_1'/C_1^\prime$ resonances of the T$_{g}$ crystal corresponds to the maximum of the distribution in the glass. For lactose, an upfield shift of the NMR distributions is observed, particularly important for the glucose residue $C_4$ resonance. For sucrose, the observed shift is downfield. These observations support the idea that, at least for lactose and sucrose, the most probable molecular conformations in the glassy state are different from those observed in the crystalline forms.

B. Energy and chemical shift surfaces

This section illustrates the results of the two steps leading to the simulations of the CPMAS spectra. First, the relaxed molecular conformations are generated by molecular mechanics, minimizing the corresponding potential energy surfaces $E(\phi, \psi)$, for each pair $(\phi, \psi)$. Then, for each conformation the corresponding $\sigma(C_\alpha, \phi, \psi)$ value of the glycosidic carbons is calculated.

Figure 2 presents portions of the potential energy maps obtained by relaxing an “isolated” sugar molecule by CHARMM-type molecular mechanics with the BIO085 parameters. Here the minimum energy conformation of the molecule constrained in each given $\phi, \psi$ pair is searched. Only the region of the map around the lowest energy basin is shown. It appears that the crystalline conformations of all three sugars, namely, trehalose, lactose and sucrose, are located within the 1 kcal/mol contour, although they are not exactly at the minimum (see crossed circles and squares in Fig. 2 and data of Table III). Because of the known variations of glycosidic angles of crystalline sugars in different poly-
morphs and crystalline forms, no major significance is given to this difference (the reader is also directed to the discussion reported in Ref. 23, concerning the unusually large shift observed in the resonances of C1 and C1' of anhydrous crystalline trehalose). However, at least qualitatively, the angular difference reported in Table III accounts for the shifts observed in the simulations. Therefore, this second procedure defines a reference to a single molecule energy landscape model, as an adjustable parameter for fitting.

\[
\chi(\phi, \psi) \propto \exp \left[ -\frac{E(\phi, \psi)}{RT_B} \right].
\]

(2)

The variable temperature has been justified in order to partially correct the above mentioned assumptions. A second simplified energy surface has been introduced with the purpose being to test the sensitivity of the procedure to major changes in the shape and position of the energy well. Therefore, a Gaussian partition of conformations was assumed, with its maximum centered on \((\phi_\alpha, \psi_\alpha)\) and width parameters \(\Delta \phi\) and \(\Delta \psi\) defined by Eq. (3),

\[
\chi(\phi, \psi) \propto \exp \left[ -\frac{(\phi - \phi_\alpha)^2}{2\Delta \phi^2} - \frac{(\psi - \psi_\alpha)^2}{2\Delta \psi^2} \right],
\]

(3)

where \(\phi_\alpha, \psi_\alpha, \Delta \phi, \Delta \psi\) were adjustable parameters for the simulations. Therefore, this second procedure defines a set of “equivalent” energy basins that provides the quantitative comparison for flexibility.

As shown in Fig. 4, the simulations with Boltzmann populations \(\chi(\phi, \psi)\) of the \(E(\phi, \psi)\) maps of Fig. 2 have been assumed by using the temperature \(T_B\) as an adjustable parameter for fitting.

**Table III.** Lowest energy conformations \((\phi_\alpha, \psi_\alpha)\) calculated from the BIO85 force field for trehalose, lactose, and sucrose, compared to the dihedral angles \((\phi_\alpha, \psi_\alpha)\) observed in crystalline forms (Refs. 23, 24, 26, 34, and 42) of trehalose, lactose and sucrose.

<table>
<thead>
<tr>
<th></th>
<th>(\alpha, \alpha)-trehalose</th>
<th>(\alpha)-lactose</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculated ((\phi_\alpha, \psi_\alpha))</td>
<td>(65°, 65°)</td>
<td>(-60°, 100°)</td>
<td>(-20°, 100°)</td>
</tr>
<tr>
<td>Crystal ((\phi_\alpha, \psi_\alpha))</td>
<td>(60.1°, 60.8°)</td>
<td>(-84.21°, 66.9°)</td>
<td>(-44.8°, 107.8°)</td>
</tr>
<tr>
<td></td>
<td>(74.78°, 61.71°)</td>
<td>(-84.93°, 85.9°)</td>
<td>(92.6°, 94.6°)</td>
</tr>
</tbody>
</table>

\(\phi_\alpha\) and \(\psi_\alpha\) are the reference points for the simulations.

\(\Delta \phi\) and \(\Delta \psi\) are the width parameters.

\(\Delta \phi\) and \(\Delta \psi\) are the adjustable parameters for the simulations.

**Note:**

- Anhydrous.
- Stable anhydrous.
- Dihydrate.
- Monohydrate.
of trehalose the assumption of a large temperature value in the Eq. (2) is able to provide a good fit of the experimental data for this system. We shall return to this point in the discussion. It has already been shown\textsuperscript{11} that the most important parameter in those calculations is the position of $\chi(\phi, \psi)$ rather than its very detailed shape. As a consequence these results indicate that either the conformational populations resulting from the molecular mechanics modeling or from the chemical shift surfaces contain approximations. Given the high apparent accuracy of the GIAO calculations, we felt that the approximation of using isolated molecules in calculating the energy surfaces was the major problem. Therefore, we sought to achieve a more effective fit of the chemical shift distributions by using more appropriate energy maps. One approach might have been to explicitly provide the environment of randomly oriented neighboring sugar molecules during the energy calculations. Instead, satisfactory agreements between experiments and calculations were obtained by using a simple Gaussian conformational probability functions $\chi(\phi, \psi)$ with their centers shifted slightly from the minima of $E(\phi, \psi)$, as reported in Table IV. Although not necessarily realistic, this result once more reveals the great influence of the highest probability region on the $S(\delta)$ profile. With such

![FIG. 3. $^{13}$C isotropic chemical shift $\sigma(C_1, \phi, \psi)$ surfaces of the $C_1$ carbons involved in the glycosidic bond of $\alpha, \alpha$-trehalose, $\alpha$-lactose, and sucrose. The maps were calculated by DFT (B3PW91/3-21+G**) on the same molecular conformations defining the energy maps $E(\phi, \psi)$. The contour lines are labeled every 2 ppm.](image)

![FIG. 4. Lower line: best fits of the experimental CPMAS spectrum (dashed line) with the chemical shift distributions for the glycosidic carbons of $\alpha, \alpha$-trehalose, $\alpha$-lactose, and sucrose, using Gaussian (solid black line) or Boltzmann (solid gray line) conformational probability $\chi(\phi, \psi)$. Upper line: corresponding best-fit Gaussian probability surfaces $\chi(\phi, \psi)$.](image)
artificial conformational distribution a good description of the experimental line shapes is obtained, except for the C4 NMR line.

At this point, it is instructive to compare the angular ranges of the conformational states that provide a reasonable fit to the experimental NMR chemical shift data (compare Tables III and IV). The Gaussian best-fit minimum remains close to the energy minimum of the single molecule $E(\phi, \psi)$ maps, and are therefore significantly shifted apart from the conformations observed in the crystalline forms. The most important conformational shifts correspond to the most important shifts observed in the NMR spectra for amorphous states, respectively. As an example, $\phi_z = -10^\circ$ for amorphous sucrose compared to a $\phi_z = -44.8^\circ$ in the crystal, which corresponds to a significant shift in the CPMAS spectrum for the C4 fructose carbon. On the other hand, still for sucrose, the angle $\psi$ seems less affected ($\psi_z = 10^\circ$ and $\psi_z = 107.8^\circ$) by amorphization, and correlatively, no important shift is evidenced in the NMR spectrum assigned to the glucose C1 carbon. Similar angular and NMR shifts are also observed for lactose, but the poor quality of the best fit obtained for the C4 glucose carbon prevents any conclusive quantitative results for this sugar.

These overall results suggest the validity of the interpretation made on the basis of the scrutiny of the experimental spectra. In other words, the average molecular conformation of disaccharides in glassy state is closer to that assumed by the isolated molecule property than to the crystalline form. This observation should be counterbalanced, however, by noticing that a single molecule description is obviously not sufficient to completely account for the actual experimental CPMAS spectra of the glassy state.

While the $\sigma(\phi_z, \psi, \theta)$ surface can confidently be assumed to be an intramolecular property, this is not true anymore for the $\chi(\phi, \psi)$ conformational occupation probability density, that can be influenced by long range interactions. As a consequence, a suitably “fitted” Gaussian $\chi(\phi, \psi)$ gives better results than the probability function provided by the force field based intramolecular $E(\phi, \psi)$.

### C. Conformational freedom of sugars in the glassy states

An interesting result of the simulations carried out with the equivalent Gaussian probabilities is the difference between the relative width of the $\chi(\phi, \psi)$ functions for trehalose and the other sugars. As reported in Table IV, and illustrated in Fig. 4, the amplitude of the probability curves (here, the standard deviation) for $\phi$ and $\psi$ torsional angles is as large as $50^\circ$ for trehalose and about $20^\circ$ for lactose and sucrose. A similar conclusion is also reached by using the $E(\phi, \psi)$ surfaces, since a quite high temperature of 1800 K had to be adopted for trehalose in the Boltzmann probability function (Table IV), although with a less satisfying fit of the experimental data. Therefore, both the Gaussian and the Boltzmann fits suggest that the conformational space explored by trehalose molecules in the amorphous phase should be larger than that calculated for the other two sugars, as well as for the isolated trehalose molecule. The same issue was raised by Zhang et al., while fitting the experimental $^{13}$C CPMAS spectrum of glassy trehalose with the energy map calculated for a model compound of trehalose. Any comments on the basis for the higher flexibility of trehalose would be pure speculation, but the practical implications deserve further discussion.

Quite a few studies have been published over time on the computation of the conformational energy of disaccharides. The central issue in these studies is the realistic representation of the glycosidic angular population and its dependence on the solvation effects. Parallel to this goal is the assessment of the role of intramolecular interactions, including the exoanomeric effect, as well as the orientation of exocyclic groups on the conformational flexibility.  

Intruding into these controversies, the results of the simple computational studies of this work are consistent with a limited conformational flexibility that should be assigned to the isolated trehalose molecule. However, trehalose appears to be an example of a disaccharide whose conformation is strongly affected by hydration or, in general, sensitive to external fields as molecular dynamics (MD) simulations at high sugar concentration show that trehalose is able to form larger clusters than sucrose. This assertion can be extended to the glassy state on the basis of the recent enthalpy relaxation studies of the three sugars during isothermal aging. It has been shown that the size of the cooperative regions in the temperature range between 298 and 365 K is much larger for trehalose than for sucrose and lactose.

A further comment is needed about the high temperature value used in the fit. As already discussed in the previous paper, this high temperature value suggests a sort of “solvent” perturbation which favors the accessibility to a larger conformational space in the amorphous state. Such a solvent perturbation has already been shown to occur in the conformational energy surfaces of maltose and cellobiose and their related polymers, amylose and cellulose, in different solvents. Therefore, trehalose molecules in the amorphous state experience a larger conformational flexibility than the isolated molecule, a fact that deserves confirmation by appropriate computational studies. A larger solvent effect has already been claimed for trehalose, in comparison to sucrose, both from experiments and from simulations. Among the experimental evidence, the diffusive properties of the two sugars in the range of concentration from dilute to fairly concentrated solutions clearly point to a higher mobility of sucrose attributed to a smaller hydration number and more compact shape. In general, a large hydration number and, therefore, the interaction with the solvent water are inferred.

### Table IV. Parameters of the best-fit populations $\chi(\phi, \psi)$ of the Gaussian form (see Fig. 4) and of the Boltzmann partition on the $E(\phi, \psi)$ maps calculated with the BIO85 force field (see Fig. 2).

<table>
<thead>
<tr>
<th></th>
<th>$\alpha, \alpha$-trehalose</th>
<th>$\alpha$-lactose</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(\phi_z, \psi_z)$</td>
<td>$(70^\circ, 70^\circ)$</td>
<td>$(-65^\circ, 125^\circ)$</td>
<td>$(-10^\circ, 100^\circ)$</td>
</tr>
<tr>
<td>$\Delta\phi = \Delta\psi$ (deg)</td>
<td>50</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>$T_\beta$ (K)</td>
<td>1800</td>
<td>300</td>
<td>300</td>
</tr>
</tbody>
</table>
from most of the experimental investigations of dilute and semidilute solutions of trehalose in comparison with other disaccharides.

The above evidence and its interpretation can be discussed in view of the interactions assessed in the crystalline structure of the three sugars and of their putative aqueous solution states. Indeed, it is easily recognized that intramolecular hydrogen bonds are present in the crystal forms as well in solution of both lactose and sucrose, while they are less relevant or absent in α, α-trehalose.23,24,26,34 The results of the present work suggest that intramolecular hydrogen bonds persist in glassy lactose and sucrose, stabilizing "compact" molecular conformations topologically close to the most probable one. On the contrary, the weaker intramolecular interactions that stabilize the isolated trehalose molecule should be easily balanced in the amorphous state by the surrounding interactions providing comparatively enhanced flexibility around its glycosidic bond.

From the evidence of the present work and from previous literature findings, the obvious conclusion concerning the enhanced flexibility of the trehalose conformation in the amorphous state is borne out.

D. Relevance to glass properties

To explore the consequence of the above reported deductions on the physical properties of the glassy disaccharides, resort is made to the well-known fragility parameter \( m \) of a glass-forming liquid and the correlation more recently established with the nonergodicity factor \( f \). The fragility \( m \) is experimentally given by the slope of \( \log(\tau) \) with respect to a normalized temperature \( T/T_g \), where \( \tau \) is the structural relaxation time and \( T_g \) is the glass transition temperature, conventionally defined as the temperature at which \( \tau = 100 \text{ s} \),

\[
m = \left. \frac{\partial \log(\tau)}{\partial (T/T_g)} \right|_{T/T_g = 1}.
\] (4)

It has been recently shown that the inverse nonergodicity factor \( f^{-1} \) (defined as the parameter \( \alpha \)) is proportional to the fragility \( m \), according to the following relation:35

\[
f^{-1}(Q \rightarrow 0, T) = 1 + \alpha \frac{T}{T_g}.
\] (5)

This factor, in the low \( T \) limit, is related to the vibrational properties of the glassy dynamics. In other words, Scopigno et al.35 claim that properties deriving from the curvature of the potential energy landscape are related to properties deriving from the viscosity increase upon supercooling. Thus, the fragility parameter \( m \) can be experimentally determined (via \( \alpha \)) from the total intermediate scattering functions, well below \( T_g \), that is in the glassy state.

Indeed, the nonergodicity parameter \( f \) can also be calculated by MD simulations. In a recent paper,36 Bordat et al. have numerically simulated the proportionality between \( \alpha \) and \( m \) for Lennard-Jones binary supercooled liquids, showing that a large fragility is correlated with the increase of anharmonicity and of intermolecular interactions within the system considered. Whether this correlation is a very general property of all materials or confined to "sticky" H-bond rich molecules has to be clarified. However, the correlation implies that, at least for homologous molecules, the change in the \( \Delta \phi \)-\( \Delta \psi \) values points to both the fragility and the nonergodicity factor.

MD simulations sufficiently long to determine the nonergodicity parameter \( f \) due to vibrations only have been carried out on trehalose and sucrose at several values of the system temperature (between 25 and 350 K), according to the method previously described.14 The results are given in Fig. 5, where the inverse nonergodicity parameter \( f^{-1}(Q \rightarrow 0, T) \) obtained from MD simulations of glassy trehalose and sucrose is plotted as a function of the temperature scaled to the respective glass transition temperature \( T_g \). The slope of the two curves of Fig. 5 in the very low temperature domain, well below \( T_g \), gives the values of \( \alpha = 0.358 \) and 0.172 for trehalose and sucrose, respectively.

Therefore, it can be inferred that the larger fragility of the trehalose glass compared to the sucrose glass is a consequence of enhanced intermolecular interactions. These enhanced intermolecular interactions take place in anharmonic potentials favoring the flexibility of trehalose, supporting the similar conclusion reached by the experimental NMR and simulations data.

E. Bioprotectant action

Some other comments appear also necessary as far as it concerns the bioprotectant role of trehalose, that is the main reason for the converging interest in the properties of this molecule. Even not going to the several hypotheses made along the last years, the central question lies in the evolution of the intermolecular interactions that trehalose is able to develop upon increasing concentration from the dilute or semidilute solution to the eventually formed solid state surrounding the biosystems to be protected.37 All findings from dilute and semidilute solutions support a privileged interaction between sugar (trehalose, in particular) and water, by displacing the “waterlike structure” in favor of a “sugar-solvation structure.”16,30 Average conformational fluctuations around glycosidic dihedral angles for different solution compositions at 200 K show little variance within the energy basin leading Dirama et al.38 to argue little influence of intramolecular hydrogen bonding on the dynamics of the system. This work provides also a reinterpretation of the “fold-
ing” of trehalose onto a biomolecule and substituting water molecules during drying by rearranging its conformation.\textsuperscript{39} However, the presence of at least one water molecule strongly bound to the trehalose molecule in solution and in hydrated mixtures seems univocally assessed by several experimental and computational investigations. Although it has been previously suggested a possible pathway in the mechanism by which water hydration is substituted by sugar-sugar interaction,\textsuperscript{13} unfortunately, the consequence of this behavior in the formation of the anhydrous sugar phase has not been fully exploited.

On the molecular level, fast rotational motions and higher rOH at $T_g$ have been taken as indications that trehalose glass is less densely packed than sucrose glass, although these molecules have the same molecular weight. This fact led those authors\textsuperscript{40} to conclude that the molecular volume of trehalose is expanded either because of the weaker intramolecular interactions or the higher molecular flexibility. The results of the present work do not provide a definitive answer to the way trehalose bioprotectant action is manifested but rather address again the attention on the subtle chameleon properties of trehalose adaptation.

**IV. CONCLUDING REMARKS**

The comparative study of the CPMAS $^{13}$C NMR experimental data of three disaccharides in the amorphous/glassy state and the numerical simulation of the line broadening of the glycosidic carbon atoms allow us to reach the following conclusions.

1. Provided that the angular dependence of the chemical shift surface is calculated with the greatest accuracy (here the density functional theory (DFT)/GIAO method), the numerical simulation is able to give a reasonable fit of the experimental data.
2. The fit of the present experimental data reveals that the sensitivity of the method relies on the gross features of the conformational energy surfaces (i.e., position and amplitude of the energy well) and not on the detail of the energy profile.
3. Fitting of trehalose data has been made possible only by imposing a probability distribution wider than that calculated within the isolated molecule approximation. This fact has been taken as an evidence of a larger flexibility of amorphous/glassy trehalose in comparison to sucrose, with some consequence on its behavior in the relaxation properties.

As a future step, it should be interesting to compare the present results with those of models that account for the cooperativity in hydrogen-bonding networks at a large length scale. In this direction, the tandem bilayer model proposed by Phillips\textsuperscript{4} for trehalose is one of the possible sketches that are compatible with our experimental and simulation data. Work is in progress in our laboratories on inelastic scattering in the UV range\textsuperscript{41} and on the possible effect of structural heterogeneity on the time scale of molecular transformations.

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