Heightened Cold-Denaturation of Proteins at the Ice–Water Interface

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ABSTRACT: The process of freezing proteins is widely used in applications ranging from processing and storage of biopharmaceuticals to cryo-EM analysis of protein complexes. The formation of an ice–water interface is a critical destabilization factor for the protein, which can be offset by the use of cryo-protectants. Using molecular dynamics simulation, we demonstrate that the presence of the ice–water interface leads to a lowering of the free-energy barrier for unfolding, resulting in rapid unfolding of the protein. The unfolding process does not require direct adsorption of the protein to the surface, but is rather mediated by nearby liquid molecules that show an increased tendency for hydrating nonpolar groups. The observed enhancement in the cold denaturation process upon ice formation can be mitigated by addition of glucose, which acts as a cryoprotectant through preferential exclusion from side chains of the protein.

INTRODUCTION

Proteins generally function optimally at physiological temperatures; however, practical considerations may make it necessary to place a protein under freezing conditions. For example, freezing is commonly used in the manufacturing process of biopharmaceuticals, with water removed from the drug through lyophilization. Freezing is also used for long-term protein storage, as well as in cryogenic electron microscopy (cryo-EM) studies and in spectroscopic studies such as double electron-electron resonance (DEER). Freezing can, however, have detrimental effects on the protein, including denaturation, aggregation, and loss of biological activity. The conformational changes that may occur during freezing are linked to cold denaturation, cryo-concentration, and formation of the ice–water interface.

Proteins experience reduced stability at extreme values of temperature; however, the effects of high and low temperatures on protein conformations are remarkably different. Heat denaturation is entropically driven, as it is mainly dictated by the increase in conformational entropy associated with protein unfolding. In contrast, cold denaturation is enthalpically driven. More specifically, at low temperature, the repulsive interaction between nonpolar residues and water is weaker, leading to a partial unfolding of the protein. Water is a key player in cold denaturation, and computational models employing an explicit description of the solvent have been applied to the study of this process. For instance, it was found that cold unfolding can be driven by the increased stability of hydrogen bonds at the protein–water interface at low temperature, with shell water molecules forming hydrogen bonds more favorably than bulk water, resulting in an overall enthalpic gain. Low-temperature unfolding occurs with an associated release of heat from the hydration shell, whereas the opposite is true in the case of heat-induced denaturation.

The sharp reduction in temperature is not the only phenomenon that may be harmful to protein stability during freezing. The ice/freeze–concentrate interface, for instance, may loosen the native structure, leading to partial unfolding. A clear experimental piece of evidence of ice-formation-induced denaturation is the direct correlation between cooling rate and loss of biological activity, with more damage found at higher cooling rates. This occurs because more rapid cooling results in smaller ice crystals, which expose a greater surface area-to-volume ratio than larger ones. In line with these considerations, solutions of the azurin protein exhibited a dramatic decrease in the average phosphorescence lifetime of the Trp-48 residue at the onset of ice formation, which is indicative of protein unfolding.

Several phenomena can therefore adversely affect protein stability during freezing, but it has been shown that ice formation represents the most critical destabilizing factor. For instance, using LDH as a model protein, a remarkable loss...
of activity was observed in frozen systems, while no
degradation was detected in concentrated solutions at the
same temperature and composition, but without ice. Hence,
prevention of ice-induced denaturation is a key consideration
whenever a protein is subjected to a freezing process.

The mechanism of ice-induced denaturation of proteins is
difficult to pin down because of the lack of appropriate
experimental techniques to address this problem. Strambini
and Gabellieri27 used intrinsic phosphorescence emission to
demonstrate that the formation of ice alters the native fold
of proteins, and suggested that this perturbation may originate
from the direct interaction between the protein and the ice
surface. They also found that the addition of cryoprotectants
such as glycerol and sucrose dramatically attenuates, or even
eliminates, the loss of structure during freezing. It was
therefore hypothesized that the stabilizing action of cosolutes
should be regarded as a combined effect of lowering the
freezing temperature and decreasing the adsorption affinity
of the protein by coating the surface of ice. It has also been
suggested28 that the preferential exclusion of the cosolutes
from the protein, which is believed to stabilize the native fold
of proteins in the bulk,21,22 may also contribute to an increased
protein stability at the ice–water interface. However, the
protective effect of these osmolytes against ice-induced
denaturation is at present poorly understood.

The present work aims to provide insight into the effect of
the ice–water surface on protein stability, and to clarify the
role of cryoprotectants. For this purpose, all-atom molecular
dynamics (MD) simulations are used. To overcome the time
scale limitations of conventional MD, we employ the
metadynamics enhanced sampling method26 in its parallel
bias variant.27 Parallel bias metadynamics (PBMetaD) allows
for a more complete sampling of the configurational state space
without the limit of requiring only a very few number of
collective variables. We also use the recent frequency adaptive
metadynamics (FaMetaD)28 method to obtain a rough
estimate of kinetic properties. As a model protein for this
investigation, we consider the 62 residue IgG-binding domain
of peptostreptococcal protein L, which has been widely studied
both experimentally29–34 and computationally.35,36 Protein L
(see Figure 1) has a native fold with both an α-helix (residue
23EKATSEAYADTL36) and a β-sheet structure (residue
2I4KANLI, 13TQTAEF18 43WTV45 , and 53TLNIKFL58), which
may be disrupted upon interaction with the ice–water surface.
The stabilizing mechanism of common cryoprotectants is then
investigated, with the aim to understand their effect at the ice–
water interface. Glucose is considered as a model osmolyte,
and its behavior at the ice surface is compared to its protective
action in the bulk.

As previously mentioned, the ice surface was experimentally
observed to promote unfolding.19–24,39,40 In line with this, we
observe in our simulations that the unfolding process is
characterized by a small free energy barrier in the presence of
ice, and proceeds with faster kinetics than in bulk. However,
experimental approaches have thus far not uncovered the
mechanisms that lead to ice-induced unfolding. In this work,
we put forth a possible explanation for these phenomena based
on our atomistic-level simulations. More specifically, we
suggest that the observed destabilization of the protein
structure at the ice surface is due to an enhancement of cold
denaturation phenomena, and is not mediated by direct
adsorption. The addition of glucose is shown to stabilize the
protein structure, because of preferential exclusion from
specific patches on the protein surface.

■ MATERIALS AND METHODS

Simulation Details. All simulations were performed using
Gromacs 5.1.441 patched with Plumed 2.4.1.38 The protein L
(topology file was obtained from the RCSB Protein Data Bank
(PDB: 2PTL39), and modified using the software Pymol to obtain
the Y43W point mutant already studied in previous experimental
works.25,26 It was demonstrated that the Y43W mutation does not
cause significant perturbations of the wild type structure,29 and
was here introduced only to allow a direct comparison with experimental
data. The OPLS-AA force field40 was used, in combination with the
TIP3P water model.41 For simulations of glucose, the OPLS-AA-SEI
force field42 was employed.

The GenIce algorithm37 was used to obtain an initial configuration
of hexagonal (Ih) ice with proton disorder and zero dipole moment,
and the generated ice layer (8.6 × 8.1 × 2.7 nm) was oriented with
the basal (0001) plane in the direction of the liquid phase (z-axis).
Afterward, the Ih ice water molecules were kept frozen in place during
the simulations.

Periodic boundary conditions were used, and the cutoff radius for both
Cowoumbic (calculated using the PME method43) and Lennard-
Jones interactions was 1.0 nm. One native protein molecule was
introduced into each simulation box, and its charge was neutralized
using Na+ ions. After energy minimization with the steepest descent
algorithm, the system was equilibrated for 5 ns at 1 bar and 260 K in
the NPT ensemble, using Berendsen pressure and temperature
coupling.45

The conformational stability of the IgG-binding domain of
peptostreptococcal protein L was investigated both in bulk and at
the ice–water interface, using parallel bias metadynamics (PBMeta-
D).27 The effect of glucose on protein stability was also addressed.
The PBMetaD simulations were then performed at 260 or 200 K and
1 bar in the NPT ensemble, controlling temperature and pressure
with the V-rescale thermostat46 and Parrinello–Rahman barostat,47
respectively. Each simulation was run for a total time of 1.0 μs, using a
2 fs time step.

Parallel Bias Metadynamics. In parallel bias metadynamics,27
multiple one-dimensional bias potentials, each acting on its own
individual collective variable (CV), are simultaneously applied. As a
result, a large number of CVs can be biased at a reasonable
computational cost. This is useful in enhancing the exploration of
phase space for systems with many degrees of freedom such as in the
case of protein conformational changes. We used a combination of the
distance d between the protein center of mass (COM) and the ice–
water interface, the protein α-helix (α), the protein antiparallel β-
sheet (β) content, and the root-mean-square deviation (dRMSD) of
the backbone atoms with respect to the reference structure as CVs.

Figure 1. Cartoon structure of the IgG-binding domain of
peptostreptococcal protein L, where the different secondary structure
elements have been highlighted using different colors: purple, α-helix;
yellow, extended β-sheet; cyan, turn; white, coil.
Convergence of the free energy surface was assessed by surfaces were computed using the reweighting technique of Tiwary et low enough to result in cold unfolding. Converged free energy temperature used in simulations 7 and 8 (200 K) was selected to be (260 K) was above the onset of cold denaturation for protein L, the concentration. While in simulations 1 molecules within the box was adjusted so as to give a 1 M water interface. In simulations 2 and 4, the number of glucose simulations 3, 4, and 8 were aimed at studying the e and 7 the bulk behavior of protein L was investigated, while

The three most sampled conformations were

about the de

time (see the SI, Figures S1–S6).

As a control, two unbiased simulations were performed in water and at the ice–water interface at 260 K, in the absence of glucose (simulations 5 and 6 in Table 1).

Kinetic Analysis of the Unfolding Process. Frequency adaptive metadynamics (FaMetaD) was used to find approximate unfolding rates of protein L at 260 K, both in the bulk and at the ice–water interface. FaMetaD35 is a variant of the metadynamics enhanced sampling method where the bias deposition frequency is adjusted during the simulation time. The bias deposition frequency is quick at the beginning of the simulation, to quickly fill up the free energy basins, but is then reduced as the system moves close to the transition state region, in order to minimize the risk of biasing the free energy barriers. Low-dimensional approximate representations of the reaction coordinate were used for this investigation. Computational details about the definition of these reaction coordinates and the kinetic analysis can be found in the SI.

RESULTS AND DISCUSSION

Protein L Is Destabilized by the Presence of Ice, while Glucose Has a Cryoprotective Effect Both in the Bulk and at the Ice Surface. Using PBMetaD simulations, we computed the free energy surfaces (FES) for protein L both in the bulk and in the presence of the ice–water interface. The presence of glucose as a model cryoprotectant was also considered. The results obtained at 260 K are shown in Figure 2, where the FES as a function of the antiparallel β-sheet content (β) and radius of gyration Rg or the α-helix content (α) and dRMSD are shown in the left and middle panel, respectively. The three most sampled conformations were identified in each FES, and labeled with a letter followed by a number. The letter N corresponds to the most folded (native-like) structure, while letters A and B were used to identify two partially folded conformations, where either the α-helix content (conformation A) or the antiparallel β-sheet content (conformation B) have been lost to some extent. The number in each label is then used to distinguish between the different simulations, as listed in the first column of Table 1.

The relative contribution in the FES as a percentage of each of these conformations was also computed as follows:

\[ P_X = \frac{\int_X d\alpha F(\alpha)}{\int d\alpha F}\]  

where \( F(\alpha) \) is the free energy, and the integral in the numerator is over a subset of the volume that defines basin X = N, A, or B. The calculated percentages are also displayed under the cartoon of each structure in the right panel of Figure 2.

260 K is still above the onset of cold denaturation for protein L. As a result, the native fold remained the most probable (>98%) in bulk water (Figure 2a), even though both the β-sheet and α-helix content could be partially lost upon unfolding. For instance, structure A1 (~1.63% probability)
showed values of α-helix content α as low as 2, and conformation B1 (~0.12% probability) was characterized by β-sheet content β = 4, compared to values of 15 and 9.5 for α and β, respectively, in structure N1 (Figure 2a).

Upon addition of glucose (Figure 2b), the FES was restricted toward more folded conformations (the relative contribution of unfolded conformations was on the order of 1.0 × 10⁻⁸% or 5.2 × 10⁻¹⁵% for A2 and B2, respectively). In this case, the β-sheet structure was remarkably stabilized, with conformation B2 still showing a significant β-sheet content (β ≈ 7.6). An almost complete loss of the α-helix content was still possible (α = 2 for structure A2), but the free energy barrier for this unfolding process was significantly higher than in bulk water. The stabilizing effect of glucose observed in our simulations is in line with the experimental results of Plaxco and Baker, where the addition of 1 M glucose was found to produce a 1 kcal/mol increase in the free energy of unfolding for protein L.

In contrast, the free energy barrier of unfolding was reduced by the presence of the ice–water interface, as shown in Figure 2c. The folded conformation N3 could in fact lose its β-sheet (β ≈ 6 or lower in structure B3, having probability 1.75%) or α-helix content (α < 5 in structure A3, having probability 8.54%) almost without free energy penalty.

Finally, Figure 2d shows that glucose has a stabilizing effect also at the ice–water surface, again restricting the FES toward more folded conformations and significantly hindering the loss of β-sheet content (β ≈ 7 in structure B4, characterized by 0.04% probability). The loss of α-helix content is still possible in the presence of glucose (α = 1 in structure A4, that shows a low probability, on the order of 10⁻⁶%), but a quite large energy barrier characterizes this unfolding process.

A lower temperature (200 K) at which cold unfolding should be more pronounced was also considered (simulations 7 and 8 in Table 1), and the results obtained in these conditions are shown in Figure 3.

In this case, the native fold was extremely unstable (<0.1%) both in bulk water and at the ice–water interface. Because of this, it was difficult to distinguish any remarkable worsening of protein stability in the presence of the ice surface. At this lower temperature, the protein could explore a wide ensemble of unfolded structures, and the identification of representative configurations for the denatured state was complicated by this extreme instability. For this reason, we will focus our next investigation on the simulations performed at 260 K, where a difference in protein behavior at the ice surface, compared to the bulk, can clearly be identified. Moreover, this also represents a realistic case study, as the denaturing effect of the ice surface has been experimentally observed to occur immediately after the formation of ice, and in temperature ranges where the protein is still stable in bulk.²⁹

It is interesting and important to note that a completely unfolded structure was never sampled in our simulations. Each protein conformation still preserved some secondary structure, either α-helix or β-sheet, and a large increase in the radius of gyration was never observed. For instance, the folded structure was characterized by Rg = 1.1 nm, and values of Rg larger than 1.5 nm were rarely sampled. This observation that the protein does not unfold completely can be explained considering that our simulations were performed at low temperature (260 or 200 K) with a relatively low metadynamics bias factor relative to the energy barrier associated with unfolding. Under these conditions, cold denaturation typically ensues. While heat denaturation is favored by the increase in conformational entropy as the protein unfolds to a largely extended conformation, cold denaturation is enthalpically driven. As a result, cold-denatured proteins are more compact, partially unfolded conformations, showing a mild form of structural loss.¹²⁻¹⁵ It is interesting to note that the presence of the ice–water interface does not alter the typical features of cold denaturation, leading to the formation of compact, partially folded conformations.

The protein segments which were mostly involved in the unfolding process were also investigated. In order to do this, the backbone RMSD of the protein conformations sampled during the simulations was computed, using N1 as reference structure (see SI, Figure S7). As previously mentioned, we focused our attention on the simulations performed at 260 K, as in these conditions it is easier to identify representative unfolded configurations, and the effect of the ice surface can more clearly be distinguished. This analysis showed that residues ¹⁵TQTAEFKTFEGATSEAY²⁰ generally were the most prone to undergo the unfolding process. This segment includes one β-strand (residues ¹⁵TQTAEF²⁰), and a large part of the α-helix (residues ²⁰EKATSEAY²⁵). However, in the presence of ice (conformation B3), the amino acid sequence ¹⁵KANLIFANGSTQTA²⁰ was also significantly involved in the loss of secondary structure. Therefore, in this case the β-strand ¹⁵KANL² also was disrupted. Finally, residues ³¹ADTLKKDNGEWT³⁴ showed a larger RMSD when glucose was added to the simulation box. This was true not only for the unfolded conformations B2 and B4, but also for the folded structures N2 and N4. This may indicate that the presence of glucose promotes the sampling of protein structures where this amino acid sequence is more expanded than in pure water. To further confirm this observation, structures N1, N2 and N4 were aligned using the STAMP (Structural Alignment of Multiple Proteins) program.⁴⁹ STAMP aligns protein structures by applying optimal rigid-body rotations and translations in order to minimize the C₆ distance between corresponding residues of each conformation. In Figure 4a the three superimposed structures are identified by different colors, and residues 33–44 are highlighted in yellow. In Figure 4b the same aligned structures are shown, but the coloring method

**Figure 3.** Free energy surface (FES) as a function of α-helix content and dRMSD for bulk-water (simulation 7, left), and the ice–water interface (simulation 8, right) at 200 K. The letters on the FES identify the native conformation, which in this case is extremely unstable. The relative contribution of the native structure to the FES was found to be lower than 0.1%, as shown under the graphs.
by Enhancing Cold Denaturation Phenomena. Having unfolded according to our RMSD analysis (residues 13–44) have been highlighted in yellow. In (b), the same image is shown, but a different coloring is used, where the blue areas correspond to structurally similar zones, and the red color indicates poor alignment.

allows identification of the zones (displayed in red) where the conformations are structurally different. Comparison of parts a and b in Figure 4 confirms that residues 33–44 are the most involved in expansion in the presence of glucose, even though the structural modification is not dramatic.

**The Ice Surface Promotes Loss of Protein Structure by Enhancing Cold Denaturation Phenomena.** Having established that the ice surface has a destabilizing effect on the protein structure by lowering the free energy barrier for the unfolding process, we turn to an analysis of the interaction of protein L with the ice surface. Remarkably, our analysis revealed that no direct interaction occurred between protein L and the ice–water surface. For instance, Figure 5a shows that the minimum distance between the protein and the ice interface generally remained quite large, both at 260 and 200 K. This was true for both the whole protein (black bars), and those amino acid sequences which were the most prone to unfold according to our RMSD analysis (residues 13–30 in red, and residues 2–16 in green). The number of protein–ice hydrogen bonds (Figure 5b) remained low during the simulations as well, with an average value of 0.0708 at 260 K and $4.22 \times 10^{-6}$ at 200 K. This means that the direct interaction between the protein and the ice surface cannot account for the observed destabilization of the protein structure, consistent with earlier work on the GB1 protein. In addition, solid-state NMR studies suggest that the hydration shell of soluble proteins does not freeze below the freezing temperature of the bulk solution. Siemer et al. observed that ubiquitin at −35 °C keeps its entire hydration shell, which prevents interaction with the ice lattice. The authors further suggest that most soluble proteins are likely to behave like ubiquitin, and that their hydration shell does not freeze until a temperature which is much lower than the equilibrium freezing value. Above this temperature, according to NMR measurements, no direct interaction is possible between the protein and the ice surface. More recently, X-ray diffraction studies of protein/ice interaction further suggested that two common pharmaceutical proteins, recombinant human albumin and a monoclonal antibody, interacted with ice crystals indirectly, by accumulating in the liquid-like layer above the ice surface, rather than by direct adsorption. Smaller protein molecules, such as lysozyme, were found to partition even further from the ice interface, in line with our simulations. We note that a different behavior from the one observed here can be found for a specialized class of proteins, antifreeze proteins (AFPs), that can directly bind to ice nuclei and prevent them from growing.

We also calculated the diffusion coefficient of the liquid water molecules in (unbiased) simulations S and 6, by least-squares fitting a straight line through the mean square displacement as function of time. According to the Einstein relation, the slope of this line should be directly related to the diffusion coefficient. We found that the diffusion coefficient was $(2.9138 \pm 0.0123) \times 10^{-5}$ cm$^2$/s in absence of ice (simulation S), while it decreased to $(1.8733 \pm 0.0084) \times 10^{-5}$ cm$^2$/s when an ice layer was added to the simulation box (simulation 6). Therefore, the presence of the ice surface slows down the nearby layers of liquid water molecules. We further verified whether this also translated into increased ordering of the water molecules. The tetrahedral order parameters $S_g$ and $S_S$ were computed for simulations S and 6, as detailed in the SI. For a perfect tetrahedron, $S_g$ and $S_S$ equal 0, while their value increases as the configuration deviates from tetrahedrality. In SI Figure S8, the average value of these order parameters as function of the z coordinate over the equilibrated trajectory (last 50 ns) is shown. In the case of simulation S (bulk water, red curve) both order parameters are significantly different from 0, indicating, as expected, absence of any ordering. For instance, $S_g$ is close to 0.25, which is the expected value for randomly arranged bonds. In the case of simulation 6 (presence of an ice layer, black curve) both order parameters are 0 in correspondence of the ice layer ($0 < z < 2.7$ nm), but their value in the liquid layers above the ice surface is similar to the case of bulk water (simulation S), indicating that these layers do not show any significant tetrahedral arrangement. Additionally, we computed the average number of hydrogen bonds between the liquid water molecules and the whole protein, or those regions that were more prone to unfold (residues 2–30 according to our RMSD analysis). Simulations 1, 3, 7, and 8 in Table 1 revealed that, at equal temperature, both the whole protein (Figure 6a) and residues 2–30 (Figure 6b) remain in the liquid state for the entire simulation time. The results refer to simulations 3 and 8 in Table 1.
interface should not be related to direct adsorption onto the residues but a good indicator of the hydrogen bond strength. We observed times more than in pure water (about 1.9 ps in simulation 5). We computed the average hydrogen bonds lifetime, formation, but these bonds were also significantly stronger. We found that, in the presence of ice, the nearby liquid water molecules did not arrange in an ordered structure, but were anyway remarkably slowed down, and could therefore form a large number of strong hydrogen bonds with specific regions of the protein. These regions show a slightly larger nonpolar surface area (the ratio of the nonpolar to total surface area $S_{np}/S$ is 0.53 and 0.52 for residues 13–30 and 2–16, respectively) if compared to the protein average ($S_{np}/S = 0.49$). As a result, these amino acid sequences, poorly hydrated in the folded structure, interact more favorably with water in the presence of ice (Figure 6), assuming an extended conformation. The ice surface therefore promotes the solvent penetration of poorly hydrated regions in the folded structure, which is a characteristic feature of the cold denaturation process.

Glucose Stabilizes the Native Structure by Being Preferentially Excluded from Specific Regions of the Protein. The mechanism of protein stabilization by glucose was further analyzed. According to the preferential exclusion mechanism,62,63 the protective osmolytes should stabilize the native state by being preferentially excluded from the protein surface. A possible way to quantify differences in determining the degree of preferential exclusion from specific patches on the protein surface is to compute the following relative distribution of glucose molecules:

$$\text{relative distribution of glucose molecules} = \frac{(n_{g}(1 \text{ nm})/n_{all}(1 \text{ nm}))_{1}}{(n_{g}(1 \text{ nm})/n_{all}(1 \text{ nm}))_{2}}$$

where $n_{g}(1 \text{ nm})$ and $n_{all}(1 \text{ nm})$ are the coordination number of glucose and water + glucose molecules, respectively, at 1 nm from the surface of patch 1 (numerator) or 2 (denominator). A value of the relative distribution parameter larger than 1 indicates that glucose interacts more with region 1 than with region 2, and vice versa.

The relative distribution parameter of glucose molecules for residues 33–44 (patch 1) over residues 2–30 (patch 2) is shown in Figure 7a. Figure 7a reveals that glucose interacted with different regions of the protein to a different extent. The relative distribution parameter showed an average value of 1.132, indicating that glucose was attracted by residues 33–44, while it interacted less favorably with residues 2–30. Interestingly, the protein region including amino acids 33–44 was more expanded upon addition of glucose than in bulk water, as evidenced by our RMSD and alignment analyses (SI Figure S7 and Figure 4). In contrast, a 1 M glucose concentration promoted a more collapsed conformation of residues 2–30, which were the most prone to undergo cold denaturation.
According to these results, we conclude that glucose stabilizes the native fold by being excluded from the hydrophobic, unfolding-prone regions of the protein. However, glucose promotes a slightly different native state, because of preferential interaction with residues 33–44. The glucose molecules may be particularly attracted by this amino acid sequence because of its hydrophilicity ($S_{np}/S = 0.43$ for residues 33–44, compared to an average value $S_{np}/S = 0.49$ for the protein and $S_{np}/S = 0.53$ for residues 2–30).

The observation that the preferential exclusion from specific amino acid sequences is key for protein structural preservation is in line with previous observations and suggests the interpretation of the preferential exclusion mechanism as a variant of the poor solvent theory for polymers. It is well known that the random coil of a polymer adopts an unfolded conformation in a good solvent, while a more collapsed conformation is sampled in a poor solvent. Proteins represent a particularly heterogeneous class of polymers, and their surface often exposes patches with extremely different features, in terms of hydrophilicity/hydrophobicity or surface charge. As a result of this heterogeneity, it is not easy to define a poor solvent for a protein as a whole, and a good solvent for specific patches on the protein surface could be, at the same time, a poor solvent for other amino acid sequences of the same protein. In this framework, glucose proved to be a good solvent for residues 33–44 of protein L, but a poor solvent for the hydrophobic region formed by amino acids 2–30.

It has also been suggested that polyols and sugars may stabilize the native fold by coating the surface of ice and decreasing the adsorption affinity of the protein. However, we did not observe a direct adsorption of protein L to the ice–water interface in our simulations (Figure 5). Moreover, Figure 7b indicates that glucose was not attracted to the ice surface, and could therefore not form a coating of adsorbed molecules at the ice–water interface. The stabilizing mechanism of glucose is therefore not related to a decreased protein adsorption, at least in the case of protein L. In contrast, the protein approached the ice–water interface similarly in 1 M glucose (Figure 7c) and in pure water (Figure 5a).

**Kinetic Analysis.** Using the frequency adaptive metadynamics approach, we can estimate the relative difference in unfolding times of the protein in the presence and absence of ice. The details of the calculations are shown in the SI. Although the unfolding times reported in the SI can only be taken as estimates, they nonetheless permit a discussion of general trends. According to our results, the unfolding process is faster at the ice surface than in bulk water even though the presence of ice slows down the motion of the surrounding water molecules. As shown in Figure 2, we observed that the ice surface promotes a significant reduction in the free energy of unfolding. The FaMetaD simulations further show that this lowering of the energy barrier translates into an extremely fast denaturation process. Hence, the process of ice formation represents a critical destabilizing factor for protein stability, from both a thermodynamic and a kinetic point of view.

## CONCLUSIONS

We have investigated the stability of protein L at the ice surface, and compared the results to the case of bulk water. We observed a significant reduction in the free energy of unfolding at the ice surface at 260 K, which translated into a fast denaturation process. This is in line with the decrease in protein stability that is experimentally observed. However, augmenting experimental results, our simulations reveal a possible explanation for this observation. We observe that the ice interface modifies the properties of the nearby liquid water molecules, slowing down their motions and promoting the hydration of the nonpolar groups of the protein. The solvent penetration of nonpolar regions is a characteristic feature of cold denaturation, which seems to be dramatically enhanced in the presence of the ice–water interface. We also showed that glucose can counteract the ice-induced unfolding process, likely by being preferentially excluded from the hydrophobic patches of the protein rather than through decreased adsorption affinity of the protein in the presence of glucose. Our simulations offer new insights into the stabilizing action of common cryoprotectants at the ice–water interface, and suggest that consideration of specific interactions between ice and the protein should not be the main, or only, concern when designing a formulation to be frozen.


