PCCP

PAPER

Cite this: Phys. Chem. Chem. Phys., 2016, 18, 21797

Molecular basis for competitive solvation of the *Burkholderia cepacia* lipase by sorbitol and urea

Ivan P. Oliveira and Leandro Martínez*

Increasing the stability of proteins is important for their application in industrial processes. In the intracellular environment many small molecules, called osmolytes, contribute to protein stabilization under physical or chemical stress. Understanding the nature of the interactions of these osmolytes with proteins can help the design of solvents and mutations to increase protein stability in extracellular media. One of the most common stabilizing osmolyes is sorbitol and one of the most common chemical denaturants is urea. In this work, we use molecular dynamics simulations to obtain a detailed picture of the solvation of the *Burkholderia cepacia* lipase (BCL) in the presence of the protecting osmolyte sorbitol and of the urea denaturant. We show that both sorbitol and urea compete with water for interactions with the protein surface. Overall, sorbitol promotes the organization of water in the first solvation shell and displaces water from the second solvation shell, while urea causes opposite effects. These effects are, however, highly heterogeneous among residue types. For instance, the depletion of water from the first protein solvation shell by urea can be traced down essentially to the side chain of negatively charged residues. The organization of water in the first solvation shell promoted by sorbitol occurs at polar (but not charged) residues, where the urea effect is minor. By contrast, sorbitol depletes water from the second solvation shell of polar residues, while urea promotes water organization at the same distances. The interactions of urea with negatively charged residues are insensitive to the presence of sorbitol. This osmolyte removes water and urea particularly from the second solvation shell of polar and non-polar residues. In summary, we provide a comprehensive description of the diversity of protein–solvent interactions, which can guide further investigations on the stability of proteins in non-conventional media, and assist solvent and protein design.

Received 16th March 2016,
Accepted 8th July 2016

DOI: 10.1039/c6cp01789d

www.rsc.org/pccp

I. Introduction

Lipases play important roles in chemical, pharmaceutical, food, and biofuel industries. They have become important because of their specificity and catalytic efficiency.[1,2] Under appropriate conditions, lipases can catalyze the hydrolysis of oils and fats and synthesis reactions, such as esterification, interesterification, alcoholysis and acidolysis.[3] This makes lipases the most important group of biocatalysts in biotechnology.[2–4] Specifically, *Pseudomonas* lipases have been widely applied as biocatalysts in the food industry, detergent formulations, and synthesis of novel chemicals, particularly in applications where enantioselectivity is of concern.[5,6] Some BCL catalyzed reactions are hydrolysis,[7,8] enantioselective synthesis of alcohols,[9] and transesterification, which is useful for the synthesis of biodiesel.[10]

The catalytic activity of enzymes may be influenced by the temperature, pH of the reaction medium, concentration of salts, and the presence of organic co-solvents.[11] It can also be modulated by an important class of molecules called osmolytes, which are small organic molecules, such as polyols and methylamines (protecting osmolytes), or urea and guanidinium chloride (destabilizing osmolytes).[12] The protecting osmolytes in aqueous solutions have interesting effects on the structure and the stability of the enzyme, protecting them from chemical and thermal denaturation.[13,14] In particular, the influence of polyols on the stability and enzymatic catalysis has been evaluated in several studies, which revealed the possibility of an increase in the enzymatic activity by regulating osmolyte concentration.[7] However, these protecting osmolytes are protein stabilizing or destabilizing agents depending on the concentrations. For example trehalose, which is considered a protecting osmolyte, destabilizes some proteins at high concentration and/or high pH.[15] These osmolytes have been tested by nature for a long time in organisms inhabiting hostile environments. Such organisms evolved selecting the chemical nature and concentrations of osmolytes to control protein stability.[12,16] Understanding the details of the stabilization mechanism promoted by these compounds has two important aspects: (1) find out how this process occurs at the molecular level in organisms; (2) propose

Institute of Chemistry, University of Campinas, Campinas, SP, Brazil. E-mail: leandro@iqm.unicamp.br

mechanisms that can assist the rational development of biotechnological processes, of both the enzyme and the solvent.

On the other side, the protein denaturing mechanism by urea is widely discussed in the literature. For instance, urea destabilization might result from its interaction with the backbone of the proteins.[17] These urea–backbone interactions might perturb intra-protein hydrogen-bonds and consequently, the secondary structure.[17] Moreover, some rationalization was tried in terms of more general physico-chemical properties of urea solutions. Indirect and direct mechanisms of destabilization were proposed from simulations of small peptides.[18] Urea could interact directly with polar residues leading to stabilization of non-native conformations. Urea can also change the structure and dynamics of water molecules, altering the solvation of non-polar residues by water.[18] Another challenge is to understand the counteracting effect of protecting osmolytes in urea–protein destabilization. There are various explanations for these stabilizing effects, some of them being the exclusion of the urea from the protein surface, or the structuration of water.[19,20] These mechanisms are, most likely, simplified classifications for complex solvation effects.[21]

Different models were proposed for the stabilization of proteins in the presence of osmolytes, which can be roughly classified into two groups: (1) the stabilization is a consequence of the direct interaction of the osmolyte with the protein. (2) The stabilization is due to the indirect stabilization of the aqueous solvation layer of the protein.[20,22] Molecular dynamics (MD) simulations are ideal tools for the investigation of these hypotheses, as they provide a molecular picture of the distribution of the solvents. These mechanisms, of course, might be dependent on the actual nature of the osmolyte (and of the protein). At one extreme, water and the co-solvent may be partitioned at the protein surface, as we have shown to occur in mixtures of water and supercritical carbon dioxide.[23] When additives and the solvent display more similar chemical properties, the balance of interactions that promote protein stabilization become more subtle. For example, MD simulations have shown that trimethylamine *n*-oxide (TMAO) is an osmolyte that promotes protein stabilization indirectly by the enhancement of the water structure.[20] This enhancement inhibits the interaction of water molecules with the amide groups of the protein, which is a destabilizing interaction.[24,25] TMAO is still a relatively hydrophobic osmolyte, and more hydrophilic ones might stabilize the protein through alternate mechanisms. The molecular basis for polyol-induced protein stability was also studied using MD simulations.[26] It was suggested that the stabilization increases with the augmentation of the polyol volume or molecular weight. Recently, MD simulations were applied to study the counteracting effect of trehalose on urea protein denaturation.[27] A model peptide was used (*N*-methylacetamide, NMA) and radial distribution functions and hydrogen bonds were computed. It was shown that NMA–water H-bonds are affected by urea. Polyols did not compete significantly with NMA–urea H-bonds. Thus, polyol induced stabilization does not appear to result from the exclusion of urea from the protein surface.

There are several methods available to increase the stability of proteins for biotechnological applications, for example, derivatization by modifying amino acids, mutagenesis, and the use of solvent additives.[28–30] The modification of the solvent has the advantage of a lower cost and technical complexity when compared with protein modification techniques. Therefore, it is more viable for practical applications.[31] For example, Rani and Venkatesu studied the stability and activity of the BM (*stem bromelain*) enzyme in solutions of various stabilizing osmolytes (glycerol, sorbitol, sucrose, trehalose) and destabilizing chemicals (urea and guanidinium chloride).[32] This enzyme has important applications in medicine due to its antiflammatory activity and antithrombotic properties.[32] The authors' results show that the stability and activity of BM are impaired by urea, and that sorbitol acts as a stabilizing agent.

Here, we study the solvation of the *Burkholderia cepacia* lipase, formerly *Pseudomonas cepacia*, in the presence of the osmolytes sorbitol and urea. These molecules are important models for protein–osmolyte interactions existing in intracellular environments and display potential practical applicability. A detailed analysis of the role of these osmolytes, and of water, in the solvation structures of the BCL enzyme, is presented. The present results provide chemical insights into the mechanisms of intracellular protein stabilization and a chemical basis for rational enzyme engineering.

II. Methods

Molecular dynamics simulations were performed starting with a crystallographic structure of the *Burkholderia cepacia* lipase (pdb id. 1YS1), with a resolution of 1.10 Å.[33] The model contains 320 amino acid residues. The complexed ligand, hexylphosphonic acid (*R*)-2-methyl-3-phenylpropyl ester, was removed from the structure. The initial configurations were built using Packmol,[34] containing the protein, water, and Na^+ and Cl^- ions to neutralize the protein net charge (with ion concentrations around 0.1 mol L^{-1}) and sorbitol, urea, or both sorbitol and urea. We opted to fix the number of molecules of all types in all systems and simulate at room pressure and temperature, instead of choosing to fix the concentrations of only part of the components, or varying the pressure to preserve all concentrations. Sorbitol and urea concentrations of the order of 1 mol L^{-1} were used, which are in the lower limit of experimental data available. This choice reduces associative interactions between solutes, which introduce additional complications for the interpretation of results, and might not be well represented by the force fields. Four systems were constructed as shown in Table 1. The systems were equilibrated as follows: (1) the solvents were relaxed by performing 1000 steps of Conjugate-Gradient (CG) minimization followed by 200 ps of MD simulations, with all the protein atoms fixed. (2) Keeping only the Cα atoms of the protein fixed, 500 CG minimization steps were performed, followed by another 200 ps of MD simulations. (3) All the protein atoms were released, and 2.2 ns of MD simulations were performed. The final coordinates and velocities of the last simulations were used to start each production run. Production runs of 40 ns were performed for each system

 This journal is © the Owner Societies 2016

Table 1 Simulated systems. Five independent simulations of 40 nanoseconds were performed for each system

		Number of molecules Concentration (mol × L^{-1})		
System	Notation	Water	Sorbitol	Urea
1	BCL–H_2O	20 800 *55.49*	0	0
2	BCL–Sor–H_2O	20 800 *49.25*	400 *0.95*	0
3	BCL–Ure–H_2O	20 800 *52.68*	0	400 *1.01*
4	BCL–Ure–Sor–H_2O	20 800 *46.74*	400 *0.90*	400 *0.90*

in the *NPT* ensemble at 1 atm and 298.15 K. The pressure was controlled using a Langevin barostat with a 200 fs period, 100 fs decay and a piston temperature of 298.15 K. Constant temperature was set using a Langevin bath with a 10 ps^{-1} damping coefficient. The CHARMM force field was used for lipase and sorbitol molecules,[35,36] and the TIP3P model was used for water.[37] Simulations were performed using NAMD,[38] and figures were produced using VMD.[39] Five independent simulations using the above protocols were performed for each system, for a total of 800 ns of simulation. The results presented are averages of these five runs.

The analysis of the solvation of the protein was performed by computing solute–solvent (g_{ss}) distribution functions.[40] These functions are similar to radial distribution functions, but the distances computed consist of the smallest distance between atoms of the solute (in this case, the protein) and the solvent, in such a way to account for the non-spherical character of the molecules. With appropriate normalization, these functions retain the same thermodynamical interpretation as standard radial distribution functions.[40] The computation of the solute–solvent distribution functions was implemented for this work within the MDAnalysis tools.[41] The normalization of solute–solvent distributions was performed by creating, for each frame of the simulation, a randomly generated set of solvent molecules with the same density of the simulated solvent. This normalization is formally correct if there are no correlations between the positions of the solute and of the solvent molecules more than those given by their interactions, as when the solute is the whole protein. If the solute is considered as a fraction of the protein (for example, when the distribution function associated only with backbone atoms is computed), the g_{ss} with this normalization must be considered only qualitatively, because indirect correlations exist, and the distribution can only be formally normalized using simulations which take into account the remaining interactions.

The survival time of protein–solvent hydrogen bonds was evaluated by computing an intermittent time-correlation function[42] consisting of the probability that a hydrogen bond is found at time t given that it was found at time zero. We computed the fraction of the hydrogen-bonds found in consecutive frames of the trajectory, which are separated by a discrete time lapse Δt. If a hydrogen bond is present for n consecutive observed frames, it contributes to the correlation at time $n\Delta t$. In our calculations, we used Δt = 1 ps.

III. Results and discussion

The simplest explanation for the solute-induced stabilization of protein structures by osmolytes is based on the partition of the solvent and solute molecules at the protein surface.[15] Stabilization has been suggested to derive from the exclusion of the solute molecules from the protein surface, that is, from preferential hydration. The indirect modification of the dynamical and structural properties of water at the hydration layer would promote the stabilization of the protein structure. For example, osmolytes could increase the solvent viscosity, thus stabilizing the protein structure, while being excluded from the protein surface and thus not effectively competing with water. On the opposite side, destabilizing solvents could interact directly with the protein, substituting water–protein and protein–protein interactions, leading to destabilization.

The partition of solute and solvent molecules on the protein surface occurs if they have very distinct polarities.[22] Here, all solvents are polar and can form hydrogen bonds. Fig. 1 shows that the distribution of sorbitol and urea is visually homogeneous, and thus there is no obvious partitioning, aggregation, or

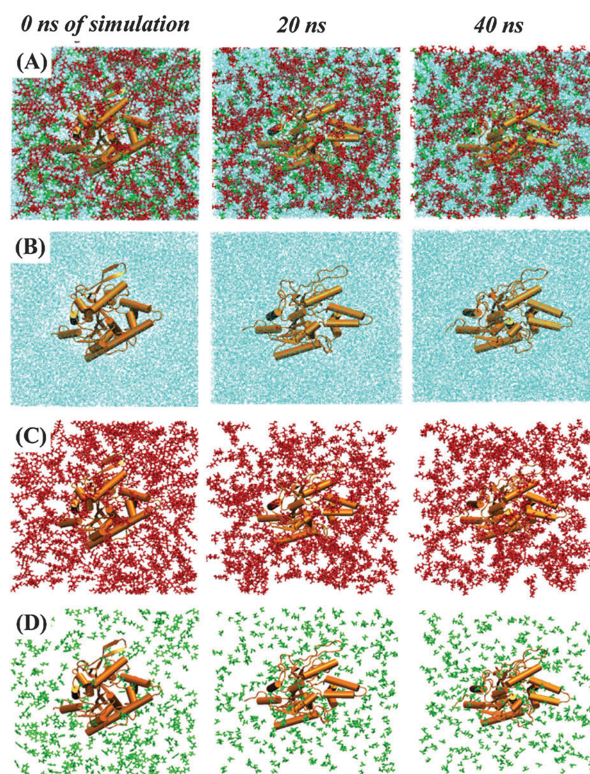

Fig. 1 Visual inspection of one of the simulations of BCL in solution of urea and sorbitol. (A) Configuration of the complete system with water (cyan), sorbitol (red), urea (green) and BCL. (B) Only water and BCL, (C) only sorbitol and (D) urea. The systems are visually homogeneous, and therefore there is no clear aggregation of sorbitol, urea or water, or any other molecular scale heterogeneity.

preferential solvation by any of the components of the mixture in any region of the protein surface. This contrasts with previous studies of enzymes in mixtures of water with less water-soluble co-solvents, in which micro-heterogeneities were observed.[23] Since there is no obvious exclusion and attraction of water or sorbitol molecules on the protein surface, the existence of preferential interactions of one or other solvent with the protein surface has to be evaluated quantitatively through distribution functions.

III.a. Protein–solvent distribution functions

The solute–solvent (g_{ss}) distribution functions[40,41] are shown in Fig. 2 for water, sorbitol and urea solvation of the protein. Fig. 2A displays the distribution of water molecules around the protein, in the absence and in the presence of the solutes. Sorbitol and urea have opposite effects on the water distribution functions: sorbitol promotes a distinct exclusion of water molecules from the second solvation shell (between ~2.5 and 6.5 Å), but promotes some organization of water at the first solvation shell, as the first g_{ss} peak increases (inset in Fig. 2A). Urea, on the other hand, causes a reduction of the first peak, indicating that it competes favorably with water for hydrogen bonds with the protein structure, but affects only slightly the distribution of water at larger distances, at least when compared to sorbitol.

Interestingly, the distribution function for water in the solution containing both sorbitol and urea is very similar to that of the sorbitol-only solution, at all distances: the first peak is essentially restored, and the density of water at intermediate distances is decreased. This means that the affinity of water for the protein surface is restored when sorbitol is added. Nevertheless, it is important to remark that the concentration of water is smaller in the mixtures with more components. Therefore, water having similar affinities to the protein with or without urea does not mean that the same number of water molecules interacts with the protein. It means that the decrease in the number of water molecules is proportional to the water molar density. This will become clear with the discussion of the potential of mean forces associated with each of these distributions.

In the scheme of Fig. 2A we try to provide a simplified view of the competing solvation effects: the solvation effects are different for the first solvation shell (corresponding to the first peak of the g_{ss} distribution functions) and further solvation shells (here considered as roughly from the first dip to about 6.5 Å). The mechanism for the exclusion of water or urea from intermediate distances is clearly associated with the volume occupied by sorbitol at the protein surface. An extended sorbitol molecule has roughly 8 Å, and thus a folded molecule will occupy volumes corresponding to distances of up to 6 Å from the surface of the protein. Thus, while sorbitol interacts through hydrogen bonds with the protein with similar affinity to water, the implied excluded volume is greater.

The addition of urea promotes a relative destabilization of water at both the first and second solvation shells, as observed by the decrease in the absolute value of the water–protein g_{ss} distribution function at all distances (Fig. 2A). Urea, therefore,

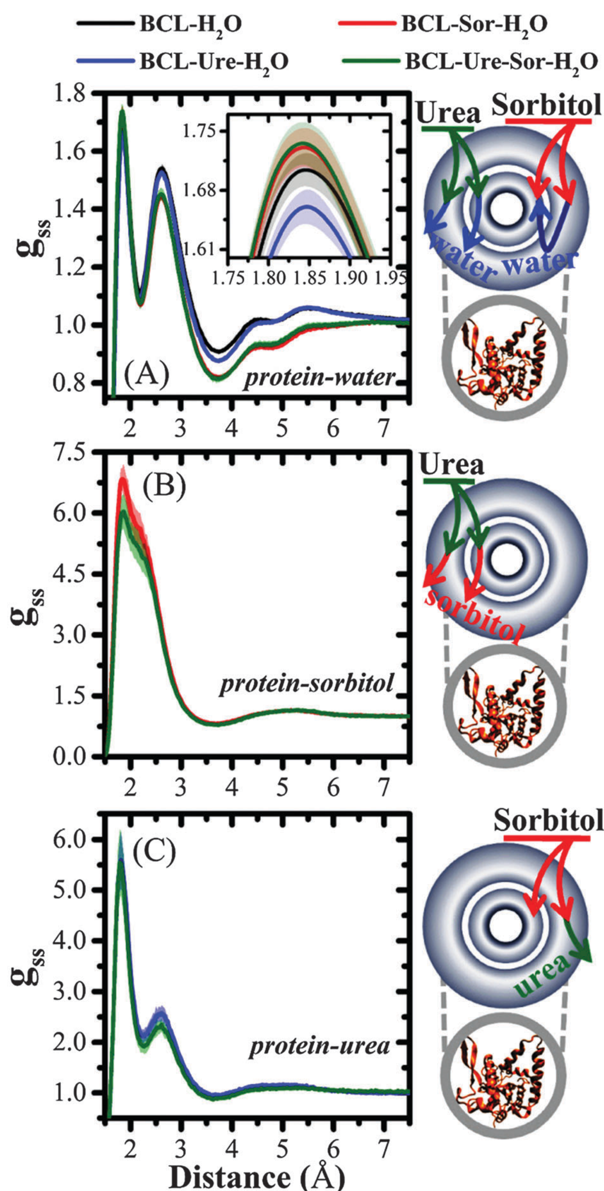

Fig. 2 Solute–solvent distribution functions (g_{ss}). The solute is the protein and the solvents are (A) water, (B) sorbitol, and (C) urea. The competition of these compounds for the protein surface is sketched on the right. In all figures, solid lines are averages computed from five independent runs, and shadow regions of the same color represent the corresponding standard deviations.

preferentially depletes water at all distances. As we will see, these effects are not homogeneous along the protein surface, and are highly dependent on the nature of the residues involved.

It is possible that stabilization of water in the first solvation layer by sorbitol contributes to stabilizing the protein. Moreover, it is clear that sorbitol promotes stabilization of water even in the presence of urea. On the other side, the addition of urea destabilizes water in both first and second solvation layers, an effect that is possibly associated with the experimental decrease in protein stability.[43–45]

 This journal is © the Owner Societies 2016

Distribution functions for sorbitol and urea molecules reveal that the competition for interactions with the protein surface is complex. As can be seen in Fig. 2B, sorbitol interacts directly with the protein surface, although the distribution function displays broad peaks, indicating that interactions are less specific than for water. The first sorbitol–protein distribution peak, at ∼1.8 Å, shows that sorbitol interacts through hydrogen-bonds with the protein. Since the first water–protein distribution function peak is slightly increased (Fig. 2A), the substitution of water by sorbitol at the protein surface occurs less than proportionally to the increase in the concentration of sorbitol in the solution. Therefore, these results show that sorbitol and water compete for the hydrogen bonds with the protein, with water having a slightly greater affinity. The addition of urea to this water–sorbitol solution (Fig. 2B, red to green curves) shows that sorbitol is displaced from the vicinities of the protein by urea. This is clear at the first solvation shell, but also at distances larger than those of the first peak. Sorbitol is, therefore, depleted from the protein surface by urea at short and intermediate distances, as indicated by the sketch in Fig. 2B.

The depletion of sorbitol by urea from the protein surface has no symmetric counterpart. Fig. 2C shows that the first g_{ss} peak of urea–protein interactions is not affected at all by sorbitol. Sorbitol does compete with urea, but only at distances greater than those of hydrogen bonds. As represented in the sketch of Fig. 2C, sorbitol permeates the first solvation layer without perturbing urea molecules, although those are destabilized at larger distances by the osmolyte.

The same distributions can be translated to potentials of mean force (PMF). Fig. 3A–C display the PMFs which correspond to the g_{ss} distributions of Fig. 2A–C. In Fig. 3A, we see that the free energy of water approach to the surface of the protein is lowest for the first solvation shell for solutions containing sorbitol, and higher for the solution of water and urea, relative to pure water. From Fig. 3B, it is clear that the affinity of sorbitol for the protein is decreased by the addition of urea, at all distances. Finally, from Fig. 3C we confirm that the addition of sorbitol to a solution of urea in water does not affect the affinity of urea at short distances, but decreases the affinity of urea for the protein at intermediate to large distances.

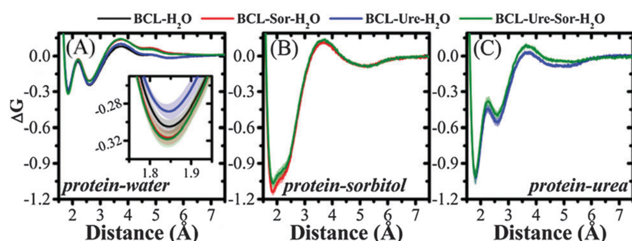

Fig. 3 Free energy profiles showing that: (A) water is stabilized by sorbitol and disturbed by urea (inset). (B) Protein–sorbitol interactions are weakened by urea at short distances. (C) Protein–urea interactions are perturbed only at long distances by sorbitol.

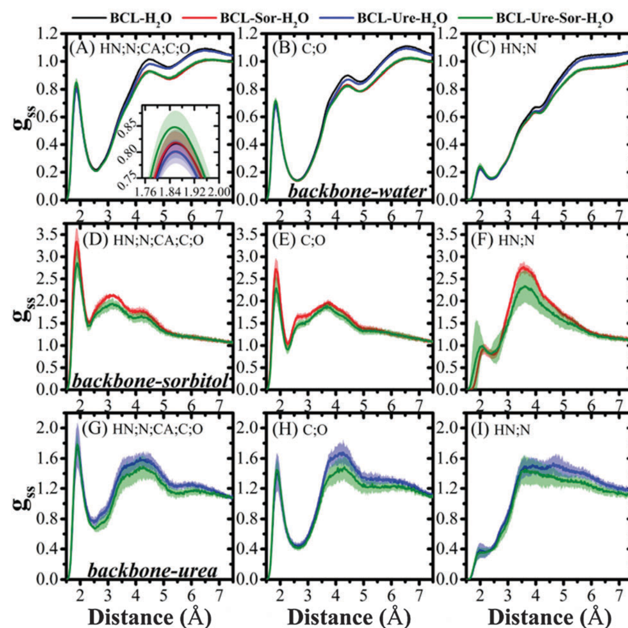

Fig. 4 Solute–solvent (g_{ss}) distribution functions of the solvents considering only protein backbone atoms. (A–C): BCL backbone and water; (D–F): BCL backbone and sorbitol; (G–I): BCL backbone and urea.

III.b. Distribution function for the protein backbone

Fig. 4 shows the distribution functions of the solvents considering only protein backbone atoms. Water can form hydrogen bonds mostly with amidic oxygens, as can be discerned from the comparison of the distribution functions from the carbonyl and nitrogen groups (Fig. 4A–C). The overall perturbation of the water distribution function by sorbitol or urea can be described similarly to that of the overall distribution functions discussed in the previous section: there is a small local density augmentation for water at short distances with the addition of sorbitol, and exclusion of water at larger distances. Urea promotes a small but consistent depletion of water at all distances. As a whole, water has a limited access to backbone atoms, in such a way that its local density is always smaller than bulk water density.

Sorbitol and urea, on the other side, accumulate in the vicinity of backbone atoms. The local density of sorbitol is almost 3.4 greater than bulk density, in pure water, at hydrogen-bonding distances, as shown in Fig. 4D. This increase in density is essentially determined by its hydrogen-bonding with the carbonyl oxygens (Fig. 4E). At the same time, there is an important accumulation of sorbitol at the second solvation shell of amidic hydrogens (Fig. 4F). The addition of urea decreases the density of sorbitol at all distances, except at hydrogen bonding distances of amidic hydrogens (Fig. 4F), ∼1.8 Å.

The local density augmentation of urea in the vicinity of backbone atoms is smaller for urea than for sorbitol. The density of urea at hydrogen bonding distances (Fig. 4G) is about 1.8 greater than bulk density (as compared to 3.4 for sorbitol). The local density of urea is reduced by the addition of sorbitol, at short distances, only in the same proportion that it reduced the density of urea on the solution, in such a way that the

distribution remains almost unchanged. At the second solvation shell and larger distances, however, sorbitol effectively excludes urea. The qualitative description of the profiles for the carbonyl and amine groups of the amide is similar, in the sense that sorbitol does not affect short-range distribution functions of urea, but excludes urea from larger distances. This exclusion is important, because it reduces the accessibility of urea to the protein backbone, which is known to disrupt protein–protein interactions.[17]

III.c. Hydrogen bonding structure and dynamics

The number of hydrogen bonds formed by the protein was computed for all systems. The distributions are shown in Fig. 5, and the average and standard deviations are shown in Table 2.

The total number of hydrogen bonds in which the protein residues participate is reduced with the addition of sorbitol, urea, or both. However, this reduction is small, of only about 4 of 278 hydrogen bonds (Fig. 5A). Interestingly, the number of protein–protein hydrogen bonds is not altered to any significant extent (Fig. 5B). This is a consequence of the fact that, within the time-scale of our simulations, no major perturbation of the protein structure was observed. Therefore, at least in the initial stages of the interaction of sorbitol or urea with the protein structure (before, in particular, urea-promoted denaturation), the most important effects concern how these additives substitute the interactions of the protein with water.

The number of hydrogen bonds of the protein with water is reduced (Fig. 5C and Table 2). The lipase forms about 206 hydrogen bonds with water in the absence of other solutes, and this number decreases to about 186 (20 H-bonds less) by the addition of sorbitol and to about 190 by the addition of urea (16 H-bonds less). Therefore, sorbitol substitutes more hydrogen bonds of the protein with water than urea (Fig. 5C). In solutions containing both urea and sorbitol, there is a further decrease of the number of protein–water hydrogen bonds, but the effect is not completely additive. Indeed, in the mixtures, sorbitol substitutes about 13 water hydrogen bonds with the protein, while urea substitutes about 10, from a total decrease in the number of hydrogen-bonds of 26. There is thus some competition between urea and sorbitol, particularly with urea displacing sorbitol molecules from hydrogen-bonding sites (Fig. 5D). Sorbitol seems to compete with urea, on the other side, but it is only capable of displacing, on average, a single urea molecule from a hydrogen-bonding position (Fig. 5E and Table 2).

The survival times[39] of hydrogen bonds performed by the BCL enzyme are depicted in Fig. 6. The addition of sorbitol promotes a small but consistent increase in the number of hydrogen bonds surviving for longer periods. Urea does not promote nearly the same effect. This can be seen in the inset of Fig. 6A, in which the fraction of hydrogen-bonds surviving for a given period are compared in the different solutions. For instance, the fraction of hydrogen bonds lasting longer (between 21 and 22 ps, for example) is 1.8 times greater in the solution of sorbitol than in pure water. It is 1.5 times greater in the solution of sorbitol and urea solution, and only 1.3 greater in the urea-only solution.

The same increase in the lifetime of H-bonds is observed for protein–protein, protein–sorbitol and protein–urea bonds, with the addition of other solvents. Fig. 6B shows that protein–protein

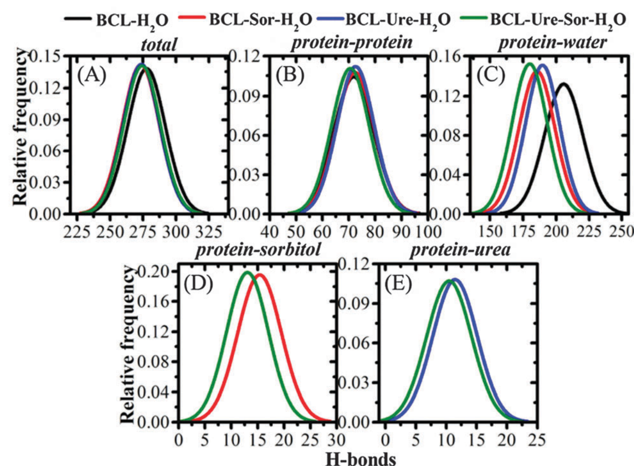

Fig. 5 Statistics of protein hydrogen bonds: (A) total; (B) protein–protein; (C) BCL–water; (D) BCL–sorbitol and (E) BCL–urea.

Table 2 Hydrogen bonds formed by the protein (standard deviations are shown)

System H-bonds	BCL–H_2O	BCL–Sor–H_2O	BCL–Ure–H_2O	BCL–Ure–Sor–H_2O
Total	278 ± 14	273 ± 14	274 ± 14	274 ± 14
Protein–protein	72 ± 8	72 ± 7	73 ± 7	71 ± 7
Protein–water	206 ± 15	186 ± 14	190 ± 13	180 ± 13
Protein–sorbitol	—	15 ± 4	—	13 ± 4
Protein–urea	—	—	11 ± 4	10 ± 4

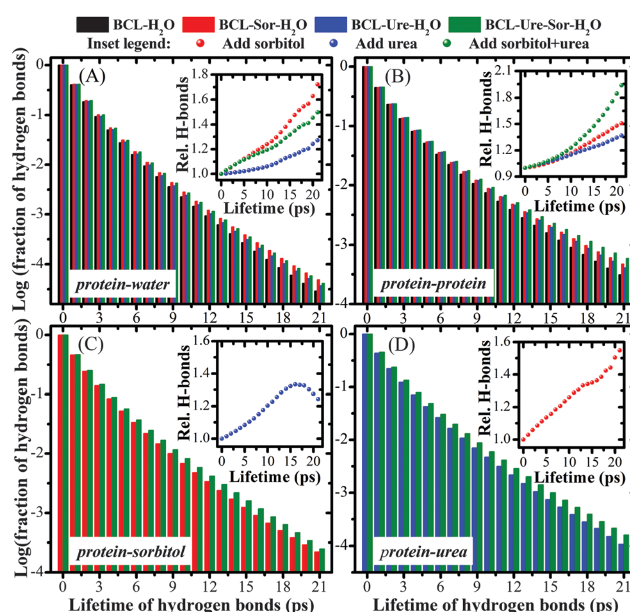

Fig. 6 Lifetime of H-bonds: (A) protein–water; (B) protein–protein; (C) protein–sorbitol and (D) protein–urea. The insets show the relative number of H-bonds calculated by dividing the lifetimes for systems with and without the addition of the osmolyte of interest.

This journal is © the Owner Societies 2016

hydrogen bonds are slightly more persistent in the presence of urea (blue), sorbitol (red), and in the mixture of both (green). The joint effect of urea and sorbitol seems to be more than additive (inset Fig. 6B). Protein–sorbitol hydrogen bonds also persist longer in the presence of urea (Fig. 6C), and protein–urea hydrogen bonds persist longer in the presence of sorbitol (Fig. 6D). In all cases, sorbitol has a more pronounced effect on the lifetimes of hydrogen bonds than urea.

The increased lifetime of water–protein hydrogen bonds resulting from the addition of sorbitol is probably related to the exclusion of water, or urea, from the second solvation shell of the protein (observed in the distribution functions of Fig. 2A and in the free energy profiles of Fig. 3). With a decreased availability of water (or urea) at the second solvation shell, resulting from the occupied volume of the larger sorbitol molecule, the hydrogen bonds are less prone to be interchanged, resulting in increased lifetimes. This could be a characteristic mechanism for stabilization of protein–water interactions, and might be one of the indirect reasons why sorbitol stabilizes the protein structure. This effect can be described as an indirect effect of the osmolyte on the water dynamics, but it is dependent on the direct attachment of sorbitol to the protein surface through hydrogen bonds, which results in the exclusion of water from the second solvation shell and vicinities because of steric effects. It is expected that this mechanism of stabilization of water–protein bonds is greater when the capacity of the osmolyte to bind to the protein surface through some specific bond is greater, as the osmolyte excludes other solvent molecules from the neighboring volume. This mechanism is interesting, because it is not completely indirect, as if depending only on the effect of the osmolyte on the properties of the solution, but it is not direct in the sense of promoting stabilization by forming specific stabilizing interactions with the protein. In other words, it is possible to suggest that sorbitol induced stabilization results from excluding both water and urea molecules from the second solvation shell of the protein.[26,46] Most interestingly, we will see that these water exclusion effects are mostly characteristic of the solvation of polar, but not charged, residues.

III.d. Protein–water distribution functions by residue types

Fig. 7 displays the solute–solvent distribution functions of water, sorbitol and urea, for each type of residue (charged, polar, and non-polar) independently. These distribution functions should be considered only qualitatively, because the normalization is not thermodynamically adequate (see Methods). Nevertheless, the distribution functions represent qualitatively the propensity of each solute or solvent molecule to interact with the different residues of the protein surface in the presence or the absence of the other solutes.

The discrimination of the solvation for each type of residue revealed that the overall distribution functions are combinations of non-trivial specific effects. In Fig. 7A, for example, the distribution of water around the protein is shown for charged residues, in the presence and the absence of the solutes. The effect of the addition of sorbitol on the first peak is, now, completely negligible,

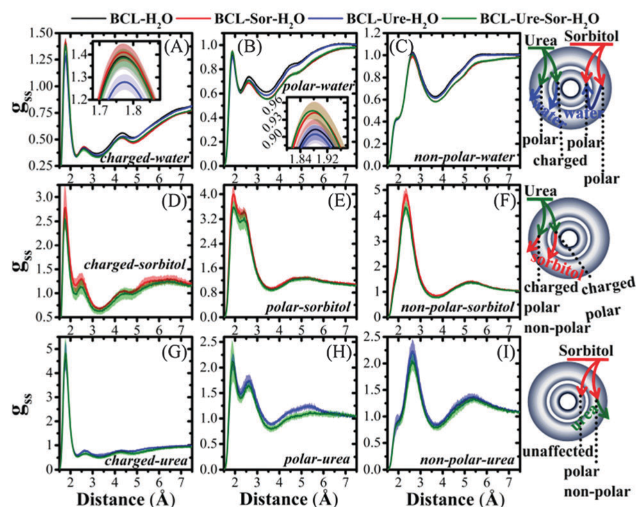

Fig. 7 Solute–solvent (g_{ss}) distribution functions of the solvents discriminated by residue types. (A–C): charged, polar and non-polar residues and water; (D–F): charged, polar and non-polar residues and sorbitol; (G–I): charged, polar and non-polar residues and urea.

in contrast to the noticeable increase it promoted in the distribution function for all residues (Fig. 2A). There is some depletion of water with the addition of sorbitol at larger distances, but the effect is also smaller than the overall decrease in g_{ss} observed for all residues. Therefore, the interactions of sorbitol with charged residues are essentially similar to the interactions of these residues with water, and substitution of water occurs only proportionally to the molar concentrations of each solvent (the relative densities at each distance are constant). Fig. 7A shows, on the other side, that the decrease of the first water–protein g_{ss} peak promoted by urea is present for charged residues. Therefore, urea substitutes water molecules at charged residues with greater affinity, decreasing their hydration more than what was expected by the decrease of its molar concentration. Finally, for the three-component mixture, the distribution is very similar to the sorbitol-only solution, both at the first peak or at intermediate distances, indicating that sorbitol indeed counteracts the effect of urea on water–protein affinity at these residues (inset in Fig. 7A).

The effect of sorbitol on water–protein distribution functions, observed in Fig. 2A, can be traced down to its effect on the distribution of water around polar residues. As can be seen in Fig. 7B, the first g_{ss} peak is increased for these residues (black to red curve), and there is a clear depletion of water at larger distances, consistent with what was observed for all residues (Fig. 2A). The same does not happen for urea: for polar residues, the effect of urea on the first water–protein g_{ss} peak is quite small. For intermediate distances, urea promotes a decrease in water affinity, as indicated by the decrease in its relative local density. Again, the distribution function of the three-component mixture is very similar to that of the sorbitol-only solution, suggesting the compensatory effect of sorbitol.

Finally, Fig. 7C shows that the effect of sorbitol and urea on the water-non-polar residue distribution functions is small,

particularly at short distances. That means that these solutes compete with water for the protein surface with the same affinity as the water molecules and substitute them only proportionally to their molar concentrations. At larger distances, sorbitol promotes some depletion of water in the vicinity of non-polar residues.

Therefore, water–protein distribution functions of Fig. 2A are a composition of different contributions from each type of residue. The most remarkable features of these compositions are:

(1) The decrease in the first water–protein g_{ss} peak with the addition of urea is mapped to the interactions of urea with charged residues.

(2) The increase in the first water–protein g_{ss} peak by the addition of sorbitol is a consequence of the greater affinity of water for polar (but not charged) residues, relative to sorbitol.

(3) The decrease in the water–protein distribution function at the second and larger solvation shells by the addition of sorbitol is due mostly to the exclusion of water molecules from the vicinity of polar residues. This exclusion is also present for charged and non-polar residues, but to a smaller extent.

(4) For the overall distribution function, but also for every residue type, mixtures of urea and sorbitol result in water–protein distribution functions which are similar to the ones observed for sorbitol-only solutions. Therefore, sorbitol restores the affinities of water to the surface of the protein, if perturbed by urea.

In the previous section, we speculated that the stabilization of water–protein hydrogen bonds by sorbitol was an indirect mechanism dependent on the direct attachment of sorbitol to the protein and the resulting exclusion of water from the second solvation shell by steric repulsion. Here, we see that this exclusion of water occurs in the vicinity of polar residues, which is consistent with its dependence on protein–sorbitol hydrogen-bonding. The effect is much smaller for non-polar residues, with which sorbitol can only hydrogen-bond to the backbone atoms, and also much smaller for charged residues, with which water interacts more strongly. We may suppose that stabilization of water molecules by sorbitol (in the first solvation shell of polar residues) aids maintaining the native protein structure. In this sense, dehydration of charged residues in the presence of urea could facilitate the denaturation process.

III.e. Competition of urea and sorbitol by residue types

Fig. 7D–I show the distribution functions of sorbitol and urea around the protein structure. These distribution functions were computed for urea-only and sorbitol-only solutions, and for the mixture containing both solutes in water. With these results, it is possible to discern the preferential solvation of each type of solute on each residue type.

Fig. 7D shows that sorbitol interacts with charged residues forming hydrogen-bonds (there is a peak at $\sim$1.8 Å). This interaction is clearly perturbed by the addition of urea (red to green curves). The same occurs for sorbitol interactions with polar (Fig. 7E) and non-polar residues (Fig. 7F), except, of course, that for the later ones there are no hydrogen-bond interactions. Urea, therefore, displaces sorbitol from the vicinity of all residue types.

Fig. 7G–I show the distribution functions of urea around each type of residue, in the absence and in the presence of sorbitol. The competition of the two solutes is not symmetrical. Sorbitol has almost no effect on the first peak of the distribution functions of urea for charged and polar residues (Fig. 7G and H, first peaks). Sorbitol does displace urea from the second solvation shell and larger distances for polar residues (Fig. 7H), as well as from the vicinity of non-polar residues (Fig. 7I).

It is possible to compare, quantitatively, the relative local density augmentation of urea and sorbitol around each type of residue. The first peak of the urea–protein interactions indicates a local density augmentation of about 4.8 (Fig. 7G). For sorbitol, the density augmentation is about 2.7 (Fig. 7D). Therefore, urea is more affine to charged residues than sorbitol. The opposite occurs for polar and non-polar residues, for which the first peaks are higher for sorbitol (4.0 and 4.9, respectively) than for urea (2.1 and 2.2, respectively). In this sense, we suggest the following effects: (1) dehydration in the charged residues is caused by the strong affinity of urea for this residue type; (2) sorbitol contribution to stabilizing the native state of proteins is due to its interaction with polar and non-polar residues.

III.f. Solvation of charged residues: acidic *vs.* basic residues

The interactions of each solvent (water, sorbitol or urea) with each type of residue are different. Urea and sorbitol, which have opposing effects on the stability of proteins, have asymmetric competing affinities for each type of residue, the most notable difference being that of charged residues. As is shown in Fig. 7D and G, urea is clearly able to compete for hydrogen-bonds with sorbitol at charged residues, but sorbitol has a much smaller relative effect on the short-range distribution functions of urea. Now, we will see that these asymmetries can be traced down to the chemical nature of each residue, with important specificities.

Fig. 8A shows the solute–solvent distribution functions of water around acidic residues (Asp and Glu). As expected, water is highly organized around these residues, due to strong hydrogen-bonding (black curve). The addition of sorbitol does not affect the water–protein distribution function, indicating that it competes with water with similar affinity, displacing water molecules in the same extent that it decreases the molar concentration of water (about 13%). Urea, on the other side (blue curve), promotes a noticeable drop of the first water–protein g_{ss} peak, and thus urea interacts with acidic residues with greater affinity than water and sorbitol. Interestingly, when both sorbitol and urea are present, the distribution functions become more similar to those of the sorbitol-only (or pure water) solutions. This suggests a counteracting effect for sorbitol, restoring the affinity of water for acidic residues. The counteracting effect is particularly clear for basic residues (Fig. 8D).

As Fig. 8B and C show, the competition of urea and sorbitol for hydrogen-bonds to acidic residues favors protein–urea interactions. In Fig. 8B it is clear that the addition of urea to a sorbitol solution displaces part of the sorbitol molecules from

 This journal is © the Owner Societies 2016

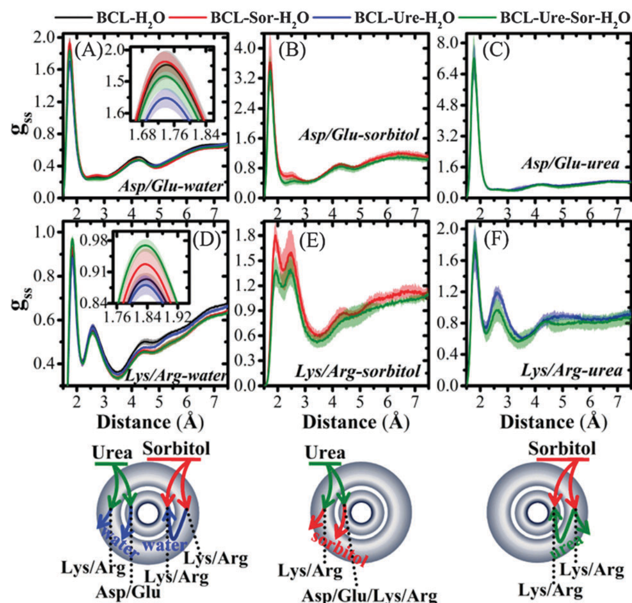

Fig. 8 Solute–solvent (g_{ss}) distribution functions of the solvents considering charged residues only. (A–C): Acidic residues and water, sorbitol and urea, respectively; (D–F): basic residues and water, sorbitol and urea, respectively; schemes showing the dynamic of exclusion promoted by each solvent.

hydrogen-bonding distances, and also from larger distances (red to green). On the other side, Fig. 8C shows that the addition of sorbitol to a urea-containing solution has no discernible effect on the organization of urea around acidic residues. It is important to note that, in particular, urea is very highly concentrated around acidic residues (the local density of urea at hydrogen-bonding distances is about seven times greater than bulk density – Fig. 8C). Therefore, urea interacts very strongly with acidic residues, and sorbitol is not able to counteract this interaction.

Fig. 8D shows that the addition of sorbitol promotes the organization of water around basic residues (Lys and Arg) at hydrogen-bond distances. This organization is even increased by the addition of urea (system BCL–Ure–Sor–H_2O). Urea competes with sorbitol for these residues, as shown in Fig. 8E. The local density augmentation for both sorbitol and urea is much lower for basic residues than for acidic residues, and is similar for each of them.

In summary, the interactions of urea and sorbitol for charged residues are different depending on whether the residues are acidic or basic. Urea interacts very strongly with acidic residues, and this interaction is not disrupted by the addition of sorbitol. Sorbitol does affect the interactions of urea with basic residues, particularly excluding it from their second solvation shell. These results clarify the importance of charged residues in the solvation structure of urea. Protein denaturation by urea might involve strong interactions of this destabilizing osmolyte with acidic charged residues. In addition, the urea induced decrease in the activity of lipases[47] can be directly explained by the inhibition of the catalytic sites, as they are dependent on Asp or Glu residues.[48,49] From this observation, we hypothesize that the mutation of charged residues at the surface of the protein to polar but not charged residues is a possible strategy to increase protein stability in media containing denaturants that act through similar mechanisms as urea.

III.g. Distribution function per atom type: preferential orientations of the solvents

In the previous sections, we have shown that urea preferentially interacts with acidic residues. Acidic residues have carboxylate groups, which should interact with urea by hydrogen-bonding the amide hydrogens. In order to obtain a detailed picture of the orientation of the solvent molecules around the protein, we computed the solvent-shell distribution functions for each atom type of solvent independently, as shown in Fig. 9.

Fig. 9A shows the distribution functions for water molecules and for each water atom type (hydrogen or oxygen) separately. The first peaks of each of these distributions show that water binds the surface of the protein by forming hydrogen bonds both through its hydrogen atoms, as a donor, or through the oxygen atoms, as a hydrogen-bond acceptor. This is, of course, expected, and no significant difference was observed in these distributions in the solutions containing sorbitol, urea, or both.

The solvent-shell distribution functions per atom, for sorbitol, are shown in Fig. 9B (no significant difference is observed in the solution containing also urea). The g_{ss} distribution for hydroxyl (pink) and oxygen (blue) atoms indicates that sorbitol forms hydrogen bonds with the protein mostly as a hydrogen-bond donor. In particular, the first peak of the oxygen g_{ss} occurs at $\sim$2 Å, while the first peak of the hydrogen distribution occurs at $\sim$1.7 Å, such that the hydrogen bonds sorbitol accept are weaker than those it participates as a donor.

The overall solvent-shell distribution function of sorbitol has a shoulder at $\sim$2.2 Å, indicating the overlap of two distinct distributions (Fig. 2B and 9B). By separating the distributions for each atom type, it is possible to attribute the distribution

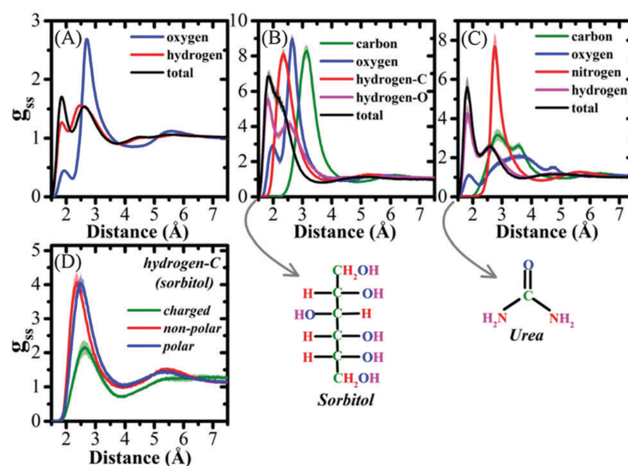

Fig. 9 Solute–solvent (g_{ss}) distribution functions discriminated by atom types. (A) Water, (B) sorbitol and (C) urea. (D) Sorbitol hydrogen-C and BCL residue type. Similar g_{ss} profiles were obtained for water in the systems with sorbitol and urea.

that forms the shoulder as the distribution of sorbitol aliphatic hydrogens (red curve in Fig. 9B – hydrogens bound to carbon atoms – hydrogen-C atoms, from now on). This indicates that this second closest-atom distribution is not resulting from an indirect, water mediated for example, interaction, but from direct non-polar interactions of sorbitol with the protein, which are not present for water or for urea. Returning to the solvent-shell distributions for sorbitol by residue types (Fig. 7D to F), we can see that the distribution that peaks at ∼2.4 Å is dominant for the interactions with non-polar residues (Fig. 7F), is important but secondary for polar residues (Fig. 7E), and has only minor relevance for charged residues (Fig. 7D). In the last case, the second peak cannot be clearly distinguished from a water-mediated interaction. Additional details on non-polar interactions can be obtained by the observation of the solvent shell distribution functions for aliphatic hydrogens separated by residue types, shown in Fig. 9D. For charged residues, the peak associated with these hydrogen atoms is after 2.5 Å and is associated with a local density augmentation of ∼2.2, which is larger than that of the second peak of the full solvent shell distribution function of sorbitol on charged residues (Fig. 7D). That second peak is also at shorter distances. This means that the aliphatic hydrogen atoms are not frequently the closest atoms of sorbitol to charged residues.

For polar and hydrophobic residues the participation of aliphatic hydrogens in the composition of the complete solvent shell distribution functions is much more important. For polar residues (Fig. 7E), the peak of the hydrogen-C distribution is closer than for charged residues, at 2.5 Å, and only slightly displaced from the shoulder observed on the total distribution (Fig. 9B). This is an indication that this shoulder is indeed associated with these non-polar interactions.

Finally, the complete g_{ss} distribution for non-polar residues (Fig. 7F) is almost completely determined by the aliphatic hydrogen distribution. There is a shoulder at short distances, corresponding to hydrogen bonds with backbone atoms, but the most significant peak and the overall curve are quite correlated with the solvent shell distribution of hydrogen-C atoms (Fig. 9D). Therefore, sorbitol forms important non-polar interactions with polar and hydrophobic residues, through its aliphatic chain. This property of sorbitol is interesting, enabling this osmolyte to interact with hydrophobic portions of the protein that are neglected by water molecules.

Atomic type distribution functions are shown for urea in Fig. 9C (the distributions are not different in the solutions containing sorbitol). The solvent shell distribution functions are mostly determined by interactions of amide hydrogen. At short distances, hydrogen bonds are also formed through the oxygen atom. No distribution for any of the atom types corresponds to the second peak of the total g_{ss}, indicating that it corresponds to the indirect interaction of urea with the surface of the protein, mediated by a water molecule.

III.h. Considerations on the perturbations of water–protein distribution functions by urea and sorbitol

A notable property of the distribution functions presented is that when both sorbitol and urea are present in the solution, the distribution functions for water are very similar to those of the solutions without urea. This means that sorbitol effectively restores water solvation free-energies, which are disfavored by the presence of urea. The interpretation of this phenomenon is not very simple, because similar distribution functions imply site occupations which are proportional to molar concentrations, and note that the solvation by water is effectively restored. In the comparison of sorbitol–water and three-component mixtures, similar water–protein distribution implicates that less water interacts with the protein surface, proportional to the reduction of the molar concentration of water resulting from the addition of urea. For short distances, this reduced number of interactions coincides with the reduced number of protein–water hydrogen bonds, shown in Fig. 5. Urea clearly displaces sorbitol molecules from hydrogen-bonding sites. The recovery of the water–protein distribution function implies, therefore, that urea is more prone to displace sorbitol molecules than water molecules. For instance, the number of hydrogen bonds between the protein and water diminishes to only ∼6 of ∼186 with the addition of urea to the water–sorbitol solution (Table 2), a ∼3% reduction. The molar concentration of water changes from 49.25 to 46.74 mol L^{-1}, thus a ∼5% reduction (the molar concentrations are approximate as they depend on an estimate of the volume of the protein). From this number, one would expect a small increase in the distribution function of water at hydrogen bonding distances, which is small but effectively observed (Fig. 2A, inset). If the same analysis is performed for the addition of sorbitol to a urea containing solution, we see that the number of hydrogen bonds is decreased from an average of 190 to 180 (∼5%), but the decrease in molar concentration of water is about 11% (from 52.68 to 46.74 mol L^{-1}). Therefore, sorbitol displaces some water molecules from hydrogen-bonding sites, but in a smaller extent than the reduction of the concentration of water. This implies an increase in the first peak of the water–protein distribution function, which is clearly visible in the inset of Fig. 2A.

IV. Conclusions

The seemingly homogeneous distribution of osmolytes sorbitol and urea around the BCL enzyme is quite complex when analyzed through distribution functions. Despite the complexity of protein–solvent molecular interactions, we can suggest some important conclusions:

(1) Overall, the protein surface is disputed by all the solvents used in the simulations: water, sorbitol and urea. The protein solvation is dynamic and heterogeneous, strongly dependent on the types of residues which consist of the enzyme and the characteristics of the atoms of the solvent molecules.

(2) Sorbitol structures water at short distances (in the first solvation shell) and displaces water at greater distances, possibly due to the sorbitol volume. Urea, on the other side, promotes the displacement of water molecules from both first and second solvation shells. Interestingly, a compensatory effect on water distributions is promoted by the addition of sorbitol in urea solutions.

 This journal is © the Owner Societies 2016

(3) There is a characteristic distribution of the solvents around the backbone of BCL: water interacts preferentially with atoms from carbonyl groups (C and O), although with lower density than in the bulk solution. Sorbitol interacts strongly with the backbone (g_{ss} peak at $\sim$2.5 Å), and this interaction is perturbed by urea. On the other hand, urea interacts at short distances with carbonyl groups. Interestingly, urea is destabilized by sorbitol only at larger distances.

(4) The hydrogen bond distributions change when sorbitol or urea (or both) is present in the BCL neighborhood. The main difference is the reduction in the number of protein–water hydrogen bonds. New H-bonds are formed between the protein and sorbitol or urea, and the total number of hydrogen bonds is essentially preserved. Sorbitol disposition around the protein surface increases the lifetime of protein–water hydrogen bonds. This can be explained by the volume occupied by the sorbitol molecules, which excludes water, preventing water exchange. This effect may be associated with the stabilizing effect of sorbitol and deserves further investigation.

(5) Water stabilization by sorbitol in the first solvation shell occurs mainly in the vicinity of polar residues. Water exclusion at larger distances occurs on all residues, but also more intensely in polar ones. Urea, in its turn, disrupts water interactions mainly with charged residues. The counteracting effect of sorbitol is observed for water distribution functions at all residue types and at all distances.

(6) Water is destabilized by urea in the first solvation shell of Asp and Glu residues. On the other hand, sorbitol stabilizes water around Lys and Arg residues. Sorbitol interactions with acidic residues are less perturbed by urea than with basic residues. Urea interactions with acidic residues are essentially insensitive to sorbitol.

(7) Finally, there is a region between the first and second solvation shells ($\sim$2.4 Å) in which sorbitol interacts favorably with the protein, through its aliphatic hydrogens. These interactions may be responsible for part of the stabilizing role assigned to sorbitol.

In summary, we have performed MD simulations to obtain a comprehensive and detailed description of the solvation structures of sorbitol and urea osmolytes on the protein surface. The results show that no simple thermodynamic model can account for the complexity of the interactions at the molecular level, and that the rational use of these data should take into account the chemical nature of residues of the protein and of the solvents. We hope that the emerging picture obtained here can guide further investigations of the stability of proteins in non-aqueous media, and allow more rational approaches for solvent and protein design.

Acknowledgements

The authors thank CAPES for a graduate fellowship, and FAPESP (grants 2010/16947-9, 2013/05475-7 and 2013/08293-7), and CNPq (grant 470374/2013-6) for financial support.

References

1 H. L. Brockman, W. E. Monsen and T. Tsujita, *J. Am. Oil Chem. Soc.*, 1988, **65**, 891–896.
2 F. Hasan, A. A. Shah and A. Hameed, *Enzyme Microb. Technol.*, 2006, **39**, 235–251.
3 P. Reis, K. Holmeberg, H. Watzke, M. E. Leser and R. Miller, *Adv. Colloid Interface Sci.*, 2009, **147–148**, 237–250.
4 N. N. Ghandi, *J. Am. Oil Chem. Soc.*, 1997, **74**, 621–634.
5 J. E. Gilbert, *Enzyme Microb. Technol.*, 1993, **15**, 634–645.
6 L. H. Andrade, L. P. Rebelo, C. G. C. M. Netto and H. E. Toma, *J. Mol. Catal. B: Enzym.*, 2010, **66**, 55–62.
7 A. Azizi, B. Ranjbar, K. Khajeh, T. Ghodselahi, S. Hoornam, H. Mobasheri and M. R. Ganjalikhany, *Int. J. Biol. Macromol.*, 2011, **49**, 652–656.
8 R. E. Fernandez, E. Bhattacharya and A. Chadha, *Appl. Surf. Sci.*, 2008, **254**, 4512–4519.
9 T. Schulz, J. Pleiss and R. D. Schmid, *Protein Sci.*, 2000, **9**, 1053–1062.
10 Q. You, X. Yin, Y. Zhao and Y. Zhang, *Bioresour. Technol.*, 2013, **148**, 202–207.
11 P. R. Burney and J. Pfaendtner, *J. Phys. Chem. B*, 2012, **117**, 2662–2670.
12 P. H. Yancey, M. E. Clark, S. C. Hand, R. D. Bowlus and G. N. Somero, *Science*, 1982, **217**, 1214–1222.
13 P. R. Davis-Searles, A. J. Saunders, D. A. Erie, D. J. Winzor and G. J. Pielak, *Annu. Rev. Biophys. Biomol. Struct.*, 2001, **30**, 271–306.
14 R. Usha and T. Ramasami, *Colloids Surf., B*, 2008, **15**, 39–42.
15 L. R. Singh, N. K. Poddar, T. Ali Dar, R. Kumar and F. Ahmad, *Life Sci.*, 2011, **88**, 117–125.
16 P. H. Yancey, *J. Exp. Biol.*, 2005, **208**, 2819–2830.
17 D. R. Canchi and A. E. García, *Annu. Rev. Phys. Chem.*, 2013, **64**, 273–293.
18 B. J. Bennion and V. Daggett, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 5142–5147.
19 *Protein Structure, Stability and Folding*, ed. K. P. Murphy, Humana Press, New Jersey, 2001.
20 J. Rosgen, B. M. Pettitt and D. W. Bolen, *Biophys. J.*, 2005, **89**, 2988–2997.
21 G. Borgohain and S. Paul, *J. Phys. Chem. B*, 2016, **120**, 2352–2361.
22 A. Kumar, P. Attri and P. Venkatesu, *Thermochim. Acta*, 2012, **536**, 55–62.
23 R. L. Silveira, J. Martínez, M. S. Skaf and L. Martínez, *J. Phys. Chem. B*, 2012, **116**, 5671–5678.
24 Q. Zou, B. J. Bennion, V. Daggett and K. P. Murphy, *J. Am. Chem. Soc.*, 2002, **124**, 1192–1202.
25 B. J. Bennion and V. Daggett, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 6433–6438.
26 F. Liu, L. Ji, L. Zhang, X. Dong and Y. Sun, *J. Chem. Phys.*, 2010, **132**, 225103.
27 S. Paul and S. Paul, *J. Phys. Chem. B*, 2015, **119**, 9820–9834.
28 M. J. Suh, D. J. Clark, P. P. Parmer, R. D. Fleischmann, S. N. Peterson and R. Pieper, *Biochim. Biophys. Acta*, 2010, **1804**, 1394–1404.

29 M. T. Reetz, S. Prasad, J. D. Carballeira, Y. Gumulya and M. Bocola, *J. Am. Chem. Soc.*, 2010, **132**, 9144–9152.
30 Y. Liu, Z. Meng, R. Shi, L. Zhan, W. Hu, H. Xiang and Q. Xie, *J. Microbiol. Biotechnol.*, 2015, **25**, 33–43.
31 Gangadhara, P. R. Kumar and V. Prakash, *Protein J.*, 2008, **27**, 440–449.
32 A. Rani and P. Venkatesu, *Int. J. Biol. Macromol.*, 2015, **73**, 189–201.
33 A. Mezzetti, J. D. Schrag, C. S. Cheong and R. J. Kazlauskas, *Chem. Biol.*, 2005, **12**, 427–437.
34 L. Martínez, R. Andrade, E. G. Birgin and J. M. Martínez, *J. Comput. Chem.*, 2009, **30**, 2157–2164.
35 O. Guvench, S. S. Mallajosyula, E. P. Raman, E. Hatcher, K. Vanommeslaeghe, T. J. Foster, F. W. JamisonII and A. D. MacKerell Jr., *J. Chem. Theory Comput.*, 2011, **7**, 3162–3180.
36 A. D. MacKerell Jr., M. Feig and C. L. Brooks, *J. Comput. Chem.*, 2004, **25**, 1400–1415.
37 W. L. Jorgensen, J. Chandrasekhar and J. D. Madura, *J. Chem. Phys.*, 1983, **79**, 926–936.
38 J. C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L. Kale and K. J. Schulten, *Comput. Chem.*, 2005, **26**, 1781–1802.
39 W. Humphrey, A. Dalke and K. Schulten, *J. Mol. Graphics*, 1996, **14**, 33–38.
40 W. Song, R. Biswas and M. Maroncelli, *J. Phys. Chem. A*, 2000, **104**, 6924–6939.
41 L. Martínez, MD Analysis. Version 15.071, Institute of Chemistry – University of Campinas, Campinas, SP, Brazil, 2016, http://leandro.iqm.unicamp.br/mdanalysis.
42 A. Luzar and D. Chandler, *Nature*, 1996, **379**, 55–57.
43 M. C. Chi, T. J. Wu, H. L. Chen, H. F. Lo and L. L. Lin, *J. Ind. Microbiol. Biotechnol.*, 2012, **39**, 1779–1788.
44 P. Venkatesu, M. J. Lee and H. Lin, *J. Phys. Chem. B*, 2009, **113**, 5327–5338.
45 M. Warepan and L. R. Singh, *Arch. Biochem. Biophys.*, 2015, **573**, 77–83.
46 D. W. Bolen, *Methods*, 2004, **34**, 312–322.
47 P. K. Chaitanya and N. P. Prabhu, *Appl. Biochem. Biotechnol.*, 2014, **174**, 2711–2724.
48 D. A. Lang, M. L. M. Mannesse, G. H. De Hass, H. M. Verheij and B. W. Dijkstra, *Eur. J. Biochem.*, 1998, **254**, 333–340.
49 Y. Cai, G. P. Zhou, C. H. Jen, S. L. Lin and K. C. Chou, *J. Theor. Biol.*, 2004, **228**, 551–557.

This journal is © the Owner Societies 2016