Structurally different chemical chaperones show similar mechanical roles with independent molecular mechanisms†

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Osmolytes are well known to protect the protein structure against different chemical and physical denaturants. Since their actions with protein surfaces are mechanistically complicated and context dependent, the underlying molecular mechanism is not fully understood. Here, we combined single-molecule magnetic tweezers and molecular dynamics (MD) simulation to explore the mechanical role of osmolytes from two different classes, trimethylamine N-oxide (TMAO) and trehalose, as mechanical stabilizers of protein structure. We observed that these osmolytes increase the protein L mechanical stability by decreasing unfolding kinetics while accelerating the refolding kinetics under force, eventually shifting the energy landscape toward the folded state. These osmolytes mechanically stabilize the protein L and plausibly guide them to more thermodynamically robust states. Finally, we observed that osmolyte-modulated protein folding increases mechanical work output up to twofold, allowing the protein to fold under a higher force regime and providing a significant implication for folding-induced structural stability in proteins.

Introduction

Protective osmolytes are small molecules that stabilize the folded state of proteins against the deleterious effects of diverse physical and chemical denaturants.1–3 These molecules share a putative stabilizing effect on proteins through a well-known osmophobic effect, the preferential exclusion of osmolytes from the protein surface. This effect allows the intrapeptide hydrogen bonds to stabilize the proteins in the folded conformation.4–6 Interestingly, few studies have contradicted this general mechanism that osmolytes could change the folding pathway and affect the unfolding transition state.4 However, osmolytes such as TMAO and glycerol are exceptional in promoting folded state stabilization without affecting the transition state.1,7,8 On the other hand, the carbohydrate osmolyte trehalose is well known to stabilize the unfolded state of proteins while destabilizing the folded state.9,10 Reports have suggested that despite this stabilizing effect, osmolytes of different structural classes interact through diverse mechanisms ranging from preferential exclusion of water bodies to separation of folding phases or even binding to the transition state, adding further complexity to their mode of action.11–16 Since the mechanical stability of proteins is an indispensable aspect of characterizing their folding phenomena, it is plausible that these osmolytes could affect protein folding by modulating their stability under force. Therefore, the molecular details of such thermodynamic preferences of different osmolytes toward the folded state of proteins are intricately context-dependent and need to be fully understood.

To address this question, we performed single-molecule magnetic tweezer spectroscopy, which employs both force-ramp and force-clamp methodologies.17,18 Using the force-ramp methodology, we increase or decrease the applied force at a constant loading rate, detecting unfolding and refolding events at different forces on a single molecule. In contrast, the force-clamp methodology at a constant applied force allows us to measure the osmolyte effect on both the folding dynamics and kinetics under equilibrium conditions. Additionally, the advantage of a larger force range of 0–120 pN allows us to inde-
pendently observe the osmolyte interactions with both the folded and unfolded states of the substrate proteins independently. Finally, using this methodology, the force can only be applied explicitly to the client protein while keeping the osmolyte molecules unperturbed.

Here, we have investigated the osmolyte effect from two different representative classes, TMAO and trehalose, on protein L as a model substrate, which has previously been extensively used in force spectroscopic studies.\textsuperscript{18–21} Our results showed that both these osmolytes mechanically stabilize protein L by increasing their unfolding force up to ∼1.3-fold and tilting the energy landscape toward the folded state. We have observed that these osmolytes decrease the unfolding kinetics and accelerate the refolding kinetics of protein L. We have explained the underlying molecular mechanism of osmolyte–protein interactions by steered molecular dynamic simulations. We found that these osmolytes boost the mechanical work output of protein L folding up to twofold by assisting their folding at higher force. Overall, this osmolyte-enhanced mechanical stability represents an interesting mechanical aspect of the folding-induced structural stability of proteins.

Results
Folding dynamics of protein L measured by single-molecule magnetic tweezers
A single-molecule magnetic tweezer assay allows us to probe the folding behavior of proteins in the presence of different external stimuli. We have experimented with a protein L polyprotein construct inserted within a C-terminal AviTag and N-terminal HaloTag. The N-terminus of the polyprotein is tethered to a glass surface via HaloTag covalent chemistry, while the C-terminus attaches with a streptavidin-coated paramagnetic bead. Force is applied by introducing a pair of permanent magnets that can exert a magnetic field vertically toward the tethered protein\textsuperscript{17–19} (Fig. 1A). The detailed method of force calibration of our instrumental setup has been previously reported.\textsuperscript{17,19} Fig. 1B shows a representative trace of protein L obtained from our single-molecule experiment. First, we applied a fingerprint pulse of 45 pN, which allows the polyprotein to unfold completely to detect the eight unfolding domains of protein L having an unfolding extension of 15 nm as a single-molecule fingerprint.\textsuperscript{18,22–24} Then, the force is quenched to 7 pN, allowing the polyprotein to refold. The minimum time it takes to unfold all the domains is termed the first passage time (FPT) for unfolding (first inset of Fig. 1B). Similarly, during quenching, the total time needed to refold all the polyprotein domains is defined as refolding FPT. Averaging such numerous trajectories, we can determine the mean-FPT (MFPT) of both unfolding and refolding kinetics as a model-free metric.\textsuperscript{19,21,25–27} Earlier studies have shown that the average (un)folding time of two-state proteins, including protein L, can be described by a single-exponential function, and the process can be defined as a Markovian stochastic process. We have explained the (un)folding time data with MFPT.\textsuperscript{28–30} FPT distributions are single-exponential to search the final state (folded or unfolded conformation) in the reaction coordinate with the MFPT as a pivotal indicator of the height of the free energy barrier and the time to conformational searching process at both the native and unfolded basins under mechanical force.\textsuperscript{31} Additionally, MFPT is used to describe the average time for stochastic processes such as protein (un)folding.\textsuperscript{26} Significantly, the MFPT analysis allows for the estimation of the average unfolding rate, irrespective of the number of steps observed in individual force spectroscopic trajectories. Moreover, MFPT denotes the briefest time leading to the complete unfolding or refolding of each trajectory, that we’ve examined in this study. Following complete refolding, the polyprotein hops between folded and unfolded states under force-induced equilibrium conditions, observed as ascending unfolding steps and descending refolding steps. From this folding–unfolding transition, we estimate the folding probability by dwell time analysis by taking many equilibrium phases from many folding trajectories. In every trajectory, displaying both unfolding and refolding MFPT, each folded domain of the polyprotein is dented as I, and their residence time along the equilibrium phase is $t = T_I / T_t$, where $T_I$ is the time expended in the I state and $T_t$ is the observable period of the equilibrium phase, characterized as $T_I = \sum_i T_i$ and $N = 8$, the total number of domains. Thus, FP is calculated as the normalized average state (eqn (1)),\textsuperscript{19,21,22,32}

$$FP = \sum_i I \times \frac{T_I}{N}$$

(1)

The folding probability is plotted as a function of force and fitted with a sigmoid equation: $p(F) = B + \frac{M}{1 + e^{-(F - F_m)/r}}$, where $B$ and $M$ are the base and maximum values of the sigmoid, respectively, $F_m$ is the force at which the half change is achieved and $r$ is the rate. From the folding probability within 8–10 pN force, we also calculated the change in folding free energy difference obtained from the equilibrium condition using eqn (2),\textsuperscript{19,33}

$$\Delta G^{0} = -kT \ln \left( \frac{FP}{1 - FP} \right)$$

(2)

TMAO increases the mechanical stability of protein L B1
We monitored the mechanical strength of protein L by force-ramp technology by increasing the force from 4 to 80 pN at a loading rate of 2.53 pN s\textsuperscript{−1}. Using the protein L polyprotein construct, we observed eight distinct unfolding steps while applying the force-ramp protocol (Fig. 2a). The unfolding extensions were vertically aligned with the force–extension curve to estimate the unfolding force of protein L (Fig. 2a, indicated by dotted line). However, during the force-decrease scan, the refolding events of protein L domains seem to be faster and smoother even in the low loading rate due to their refolding within the same force range, and thus, no distinct refolding steps could be observed (ESI Fig. 1†).
We systematically explored the effect of TMAO on the mechanical stability of protein L by observing their unfolding force using force-ramp technology. We measured the probability distribution function of unfolding forces at different TMAO concentrations. Interestingly, we observed that the most likely unfolding force increased with TMAO concentration (Fig. 2b). In Fig. 2c, the most likely unfolding force is plotted against the TMAO concentration, and the unfolding force changes negligibly up to 1 M TMAO; however, it increases significantly upon increasing its concentration to 1.5 M. These data suggest that TMAO significantly increases the mechanical strength of protein L by increasing their most likely unfolding force, plausibly indicating that TMAO may interact with the folded state of the protein L.

**TMAO modulates protein L folding events in a concentration-dependent manner**

To determine how TMAO affects the folding events of protein L, we investigated the unfolding and refolding MFPT of protein L at different TMAO concentrations. Fig. 3A and B
demonstrate the changes in refolding MFPT with TMAO concentrations at 5.5 and 6.5 pN forces, respectively. We observed that MFPT refolding decreased with TMAO concentration. Similarly, unfolding of the MFPT increased with TMAO concentration at different unfolding forces (Fig. 3C and D). For example, at 40 pN, protein L unfolding takes 13 ± 1.3 s without TMAO, whereas it increases to 71.8 ± 5.6 s with 3 M TMAO (Fig. 3D). Although we found visible differences in MFPT values, they were not statistically significant at all TMAO concentrations, as observed by one-way ANOVA, followed by the Bonferroni post hoc test at *p < 0.05 (ESI Fig. 2†). These results illustrate that TMAO slows unfolding kinetics and accelerates refolding kinetics in a concentration-dependent manner.

Furthermore, we measured the folding probability (FP) of protein L in the presence of TMAO over the range of 4 to 14 pN. We observed a drastic change in the folding probability within the intermediate force region of 7–11 pN, with the most pronounced TMAO effect at 9 pN. The half-point force (where FP = 0.5) was increased from 7.7 pN in the absence of TMAO (control) to 10.2 pN at 3 M TMAO. This upshift in FP with TMAO indicates an increased folding ability of protein L under force (Fig. 3E and Fig. 4B). However, we did not observe such an effect on protein L FP at a very low TMAO concentration (ESI Fig. 3†). Additionally, Fig. 3D demonstrates the comparative change in free energy difference within an 8–10 pN force, where the protein L polyprotein exhibits folding-unfolding dynamics under force-induced equilibrium conditions. At a 9 pN force, the calculated value ΔG⁰ of protein L folding is 1.81 kT in the absence of TMAO, whereas it decreases to −1.26 kT at 3 M TMAO. The ΔΔG⁰ (9 pN) is (ΔG_{3M}⁰ − ΔG_{0M}⁰) −3.07 kT, implying a favored folding process in the presence of TMAO.
Trehalose promotes the mechanical stability of protein L

Previous studies have shown that the effect of osmolytes on protein folding depends on their intermolecular interactions, which are intricately related to the osmolyte structure.\textsuperscript{1,35–38} Therefore, we have further extended the study to another osmolyte, trehalose, which is a polyol in nature and structurally different from methylamine TMAO (zwitterionic). Using
force-ramp technology, we measured the mechanical stability of protein L with trehalose and observed a significant increase in its most likely unfolding force from 38.6 ± 0.5 to 49.3 ± 0.7 pN in the presence of 1 M trehalose (Fig. 5A). However, the unfolding force became saturated upon further increasing the trehalose concentration up to 2 M (ESI Fig. 4†). Since kinetic partitioning is an indispensable factor mediating the osmolyte effect on protein stabilization, we also measured the unfolding and refolding MFPT of protein L in the presence of trehalose. Similar to TMAO, we found that 1 M trehalose retards the unfolding of the MFPT by ~2-fold while increasing the refolding kinetics (Fig. 5B–E). We did not observe statistically significant MFPT differences at any concentration; however, the differences between the control and 1 M trehalose were significant in all the force ranges, as observed by the Bonferroni post hoc test at *p < 0.05 (ESI Fig. 5†). Additionally, from the equilibrium condition, we observed that trehalose decreases the ΔG° of protein L folding (Fig. 5G) by increasing the folding probability (Fig. 4A), eventually shifting the half-point force from 7.7 to 9.7 pN (Fig. 5F). While studying these osmolyte effects on protein L folding dynamics under force-induced equilibrium conditions, we observed that these osmolytes do not have any significant effect on protein L extension at different concentrations (ESI Fig. 6†), and the data are also in good agreement with the WLC estimates of force-dependent extension (ESI Fig. 7†).

Osmolytes mechanically stabilize protein L through different molecular mechanisms

To decipher the molecular insight into how osmolytes of these two different representing classes modulate protein L (un)folding kinetics, we performed all-atom steered molecular dynamics (SMD) simulations of protein L both in the absence and presence of TMAO and trehalose as cosolvents, i.e., only protein L, protein L + trehalose, and protein L + TMAO. In contrast to the empirical unfolding measurement at 45 pN, we used constant velocity pulling at 0.01 Å ps⁻¹ in NAMD 3.0 using the CHARMM36 force field and TIP3P water model to

Fig. 4 Osmolyte effect on protein L folding dynamics: (A) effect of trehalose: representative trace for protein L is shown in the absence and presence of 1 M trehalose. After unfolding the polyprotein at an unfolding force of 45 pN, we refolded it at 8 pN and observed the folding–unfolding dynamics of the protein. The folding probability of protein L increased from 0.3 to 0.85 in the presence of trehalose. (B) TMAO-mediated change in the folding dynamics of protein L: a representative trace for protein L in the presence of 3 M TMAO. First, we unfolded the polyprotein construct at an unfolding force of 45 pN, and subsequently, a quenching pulse of 8 pN was applied to observe the folding dynamics at the equilibrium state. It has been observed that the folding probability of protein L increases from 0.3 to 0.9 in the presence of 3 M TMAO. The experiments were performed with 5 nM protein L.
check these osmolyte effects on protein L mechanical stability (Fig. 6). In each condition, triplicate simulations were run with the solid lines representing smoothed mean force values. We took the TMAO bond parameters from a study by Ganguly et al. and that of trehalose using CHARMM GUI software.\textsuperscript{39,40} We used the B-chain of PDB ID 1 HZ6 as the protein L B1 structure for simulation. We kept the protein in a 10 nm cubical box for control and trehalose and a 13 nm cubical box for TMAO. The RMSD (root mean square deviation) of the protein complex with different osmolytes was measured and found to be reduced in their presence, which indicates that osmolytes compact the protein structure by decreasing the RMSD, albeit to a different extent (Fig. 7). Additionally, while measuring relaxation with different osmolyte concentrations, we observed that the end-to-end distance of protein decreases maximally in the presence of 3 M TMAO compared with that of 1 M TMAO, signifying accelerated compaction of protein with 3 M TMAO within 25 ns (Fig. 8). To gain an in-depth understanding of the osmolyte-modulated relaxation phenomena, we measured the changes in the fraction of native contacts to

Fig. 5 Trehalose modulates the folding mechanics of protein L: (A) effect on mechanical stability: similar to TMAO, trehalose increases the mechanical stability of protein L by increasing their unfolding force. The probability density function of the unfolding force population both in the absence (top) and presence (bottom) was fitted to the Gaussian equation, and it was observed that the most likely unfolding force increased from 38.6 ± 0.5 pN without trehalose (top, gray bars) to 49.3 ± 0.7 pN in the presence of trehalose (bottom, yellow bars). (B and C) Trehalose modulates refolding kinetics: similar to TMAO, trehalose decreases the refolding time at 5 and 7 pN forces in a concentration-dependent manner. At 7 pN, the refolding time difference was statistically significant between the control and three different trehalose concentrations. However, a significant difference is only observed between the control and 1 M trehalose at a 5 pN force, suggesting that the refolding MFPT becomes less concentration-dependent at lower forces. (D and E) Unfolding kinetics by trehalose: trehalose has been observed to increase unfolding MFPT at 40 and 50 pN. Both of these MFPT data indicate that trehalose also stabilizes the folded state of protein L. The statistical significance has been tested by one-way ANOVA, followed by Bonferroni post hoc test at *p < 0.05 level. (F) Effect on folding probability: trehalose shifts the folding probability to a higher force range, with an increase in the half-point force from 7.8 pN (control) to 9.7 pN (with 1 M trehalose). (G) $\Delta G^0$ of folding: trehalose decreases the $\Delta G^0$ of folding in a concentration-dependent manner compared with its absence, which eventually stabilizes the folded state of protein L. The experiments were performed with 5 nM protein L.
be increased more spontaneously with osmolytes than without them (Fig. 9B). Error analysis of all the simulation data was performed from triplicate simulations (ESI Fig. 8†). Overall, these data signify that these osmolytes, although to different extents, mechanically stabilize the native structure of protein L.

**Mechanical work done by protein L in the presence of TMAO**

It is well established that protein folding generates mechanical energy for performing different cellular processes.32,41–44 From our single-molecule data, we observed that TMAO and trehalose modulate the mechanical work of protein L folding by tilting the energy landscape toward the folded state. The folding work is measured as the product of the force and step size at that particular force, as described previously.32,41 This measurement indicates that protein folding at a larger force generates more mechanical energy than the lower ones; however, FP values decrease with the force, which underestimates the work done calculation. Therefore, we precisely measured it by multiplying the folding work with the FP under a particular force. Although TMAO or trehalose do not affect
the protein L step size, they increase their intrinsic folding ability (or FP) under force, which prominently increases work done during protein L folding. We observed that protein L generates a maximum peak of mechanical work of 33.5 ± 5.8 zJ at 6.5 pN (Fig. 10B, red circles), whereas 3 M TMAO can produce mechanical energy up to 67.3 ± 15.8 zJ at ∼8.3 pN (Fig. 10B, blue circles). A similar upshift in the mechanical energy with an increased force regime was also observed with 1 M trehalose (Fig. 10A). Thus, these osmolytes can contribute ~2-fold extra mechanical work performed by the protein L itself.

Discussion

Our magnetic tweezers observation reveals that osmolytes favor protein L folding by increasing the protein L unfolding
force up to 1.3-fold, thereby promoting their mechanical stability (Fig. 2C and 5). Although we observed this significant increase at 3 M TMAO, we observed a nonsignificant change in unfolding force up to 1 M TMAO, which is consistent with an optical tweezer study by Motlagh et al.45 Previously, experimental and theoretical studies have used these osmolytes at higher concentrations (up to 5 M for TMAO and 2 M for trehalose) to study their physicochemical effect on protein stabilization.3,35,46–50 Furthermore, we measured the change in the protein folding kinetics. We observed that both TMAO and trehalose accelerate the refolding kinetics and retard the unfolding kinetics of protein L under force. This modulated kinetics plausibly reshaped the mechanical folding landscape of protein L by stabilizing its folded state and destabilizing the unfolded state (Fig. 3E, F, and 5F, G). This osmolyte-modulated reshaping of the folding landscape enhances their kinetic protection mechanism by accelerating their molecular compaction. TMAO increases the mechanical stability of native protein L while interacting with osmolyte molecules (ESI Fig. 14A†). We performed a one-way ANOVA and observed that the unfolding forces at different states were statistically significant at *p < 0.05 (ESI Fig. 14B and C†). The slower unfolding rate (kinetic protection) of protein L coincides with the indirect osmophoric effect, where these osmolytes prevent a large number of backbone-solvent hydrogen (H) bonds in the unfolded state, thereby destabilizing them.5,51 This might favor the backbone–backbone intrapeptide H-bond in protein L and preferential exclusion of osmolytes from the peptide surface to facilitate their compaction.36,51–53 Due to the simple topology of protein L with low-fold complexity (lack any folding or unfolding intermediates), it is expected that there would be very less chance of a solvent-bridging mechanism of these osmolytes, despite the presence of β-strands in their structure. Plausibly, these osmolyte interactions mediate structural rearrangement in the protein (ESI Fig. 15†) and more stabilizing their native state against the mechanical force. Overall, from the energy landscape perspective, this finding concludes that osmolytes enhance substrate mechanical stability by modifying their energy landscape, and consequently, the mechanical energy generated during this process could have plausible implications in deciphering diverse protein folding pathways.

Materials and methods

Expression and purification of protein L

We used the protein L construct as a model substrate protein, as described previously in a force spectroscopic study.18,21,54 For expression, the constructs were transformed into E. coli BL21 (DE3) component cells and grown in LB with carbenicillin at 37 °C until the O.D. becomes 0.6–0.8 at 600 nm.18,55,56 The cultures were then induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG, Sigma Aldrich) and incubated overnight at 25 °C. Then, the cells were pelleted at 9000 rpm and subsequently resuspended in 50 mM sodium phosphate buffer with 300 mM NaCl and 10% glycerol at pH 7.5. The dissolved pellet was incubated with phenylmethylsulfonyl fluoride and lysozyme at 4 °C for 20 minutes. The cell pellet was then treated with DNase, RNase, Triton-X and MgCl2 and kept in the rocking platform at 4 °C. Then, the cell pellet was disrupted in a French press at 19 psi followed by centrifugation at 4 °C. The supernatant was collected, and the protein was purified with Ni2+-NTA column chromatography (AKTA Pure, GE Healthcare). Imidazole (20 mM) was used with sodium phosphate buffer to bind the protein with the column, and 250 mM imidazole was used for elution. The polyprotein was biotinylated with a biotinylation kit (avidity) and kept at 4 °C over-

Fig. 10 Osmolyte-induced stabilization of protein L: (A) mechanical work done generated by protein L folding in the presence of trehalose: the value of mechanical work done is calculated as a product of folding work and folding probability, where the expected value of folding work is calculated by multiplying step size by force. The highest mechanical energy obtained from Gaussian fitting is 33.5 ± 5.8 zJ without trehalose (black square), while the mechanical energy increases up to 64.5 ± 0.5 zJ in the presence of trehalose (green square). (B) Similarly, the TMAO interaction evidently increases the intrinsic folding ability of protein L, which increases the mechanical energy up to 67.3 ± 15.8 zJ (blue circles).
Glass chamber preparation and magnetic tweezer experiment

For the magnetic tweezers experiment, the glass slides were cleaned with Hellmanex III (1.5%) solution (Hellma analytics) and washed with double distilled water. Then, the glass slides were treated with a mixture containing methanol (CH₃OH) and concentrated hydrochloric acid (HCl) followed by concentrated sulfuric acid (H₂SO₄). The glass slides were boiled gently in double distilled water and dried. Then, the slides were put into an ethanol solution of 1% (3-aminopropyl)trimethoxysilane for 15 minutes, washed gently with ethanol to remove the unreacted silane from the chamber and dried for 1 hour. In a very similar way, coverslips were washed with Hellmanex III (1.5%) solution and then treated with ethanol. The chamber was prepared by sandwiching the glass slides and the coverslips with parafilm. Then, the chamber was filled with glutaraldehyde (Sigma Aldrich) solution followed by reference beads (2.5–29 μm, Spherotech, AP-25-10) and O4 ligand (Promega, P6741). The glass chambers were flushed with blocking buffer (150 mM NaCl, 20 mM Tris·HCl, 2 mM MgCl₂, 0.03% NaN₃ and 1% bovine serum albumin, pH 7.4) at room temperature to block nonspecific interactions.17,19

Our custom-made magnetic tweezers were built on an inverted microscope attached with a nanofocusing piezo actuator, and images were collected with a ximea camera (MQ013MG-ON).18 Detailed information regarding image processing, bead tracking and force calibration has been described in our previous works.19 We performed a magnetic tweezer experiment with 5 nM protein L in PBS buffer at pH 7.4. Streptavidin-coated paramagnetic beads (Dynabeads M-270, cat. no. 65305) were passed through the chamber to attach the biotinylated AviTagged protein L. By applying different force-clamp and force-ramp protocols, we observed different folding and unfolding dynamics of protein L. For our experiment with TMAO, we used different concentrations of TMAO with protein L to check the change in the folding properties of the protein substrate.

Computational analysis of osmolyte–protein interactions

The crystal structure of protein L (PDB ID: 1 HZ6) was obtained and refined for simulation using the PSFgen module in NAMD. The system was solvated using the TIP3P water model in a cubic box with periodic boundary conditions, and sodium and chloride ions were added for system neutrality. The CHARMM36 force field was employed for both the protein and the chemical chaperones. The system underwent energy minimization and equilibration as implemented in NAMD, including a 100 ps NVT equilibration and a 100 ps NPT equilibration at 310 K and 1 atm. The simulations were divided into three steps per condition: equilibration, unfolding (constant-velocity pulling) and relaxation. The Co atoms of specific residues in protein L were selected for pulling in NAMD. A constant-velocity spring with a pulling rate of 0.01 Å ps⁻¹ was applied to induce unfolding. The simulation was run for 25 ns with a time step of 2 fs. The simulation box dimensions were set to 10 × 10 × 10 nm using the periodic cell setup in NAMD for trehalose systems and the control. For TMAO systems, simulation box dimensions were set to 13 × 13 × 13 nm with the periodic boundary conditions applied. In all these cases, the ultimate goal was to obtain an ultimate chemical chaperone concentration as specified (absent or 1 M or 3 M) (ESI Table 1†). Relaxation simulations were performed by withdrawing the force from the systems. To ensure the reliability of the results, triplicate simulations were performed, demonstrating consistent results throughout most of the analyses. NAMD-generated trajectories were analyzed using VMD and custom scripts. Statistical analysis in NAMD involved calculating means and standard deviations for relevant observables such as forces and extensions. Error bars were determined based on the standard error of the mean.

Author contributions

S. H. designed the project. D. C., A. D., A. M., and M. B. performed the experiment. D. C., S. C., and M. B. analyzed the experimental data. D. C., R. C. performed and analyzed the simulation study. S. H., S. C., D. C., D. C. wrote the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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