



**HAL**  
open science

# Specific Interactions and Environment Flexibility Tune Protein Stability under Extreme Crowding

Fabio Sterpone, Marina Katava, Guillaume Stirnemann, Maria Pachetti,  
Simone Capaccioli, Alessandro Paciaroni

► **To cite this version:**

Fabio Sterpone, Marina Katava, Guillaume Stirnemann, Maria Pachetti, Simone Capaccioli, et al.. Specific Interactions and Environment Flexibility Tune Protein Stability under Extreme Crowding. Journal of Physical Chemistry B, 2021, 125 (23), pp.6103-6111. 10.1021/acs.jpcc.1c01511 . hal-03364607

**HAL Id: hal-03364607**

**<https://hal.science/hal-03364607>**

Submitted on 11 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Specific Interactions and Environment Flexibility Tune Protein Stability Under Extreme Crowding.

Marina Katava,<sup>†</sup> Guillaume Stirnemann,<sup>†</sup> Maria Pachetti,<sup>‡</sup> Simone Capaccioli,<sup>¶</sup>  
Alessandro Paciaroni,<sup>\*,§</sup> and Fabio Sterpone<sup>\*,†</sup>

<sup>†</sup>*Laboratoire de Biochimie Théorique, IBPC, CNRS UPR9080, Univ. Paris Diderot,  
Sorbonne Paris Cité, 13 rue Pierre et Marie Curie, 75005, Paris, France*

<sup>‡</sup>*Elettra-Sincrotrone Trieste, S.S. 14 km 163.5, Area Science Park, 34149, Trieste, Italy.  
Department of Physics, University of Trieste, Via Valerio 2, 34127, Trieste, Italy*

<sup>¶</sup>*Dipartimento di Fisica, Università di Pisa, largo Pontecorvo 3, 56127 Pisa, Italy, and  
CISUP, Centro per l'Integrazione della Strumentazione dell'Università di Pisa, Lungarno  
Pacinotti 43, I-56127 Pisa, Italy*

<sup>§</sup>*Dipartimento di Fisica e Geologia, Università di Perugia, via A. Pascoli, 06123 Perugia,  
Italy*

E-mail: [alessandro.paciaroni@unipg.it](mailto:alessandro.paciaroni@unipg.it); [fabio.sterpone@ibpc.fr](mailto:fabio.sterpone@ibpc.fr)

## Abstract

Macromolecular crowding influences protein mobility and stability *in vivo*. A precise description of the crowding effect on protein thermal stability requires the estimate of the combined effects of excluded volume, specific protein-environment interactions, as well as the thermal response of the crowders. Here we explore an ideal model system, the lysozyme protein in powder state, to dissect the factors controlling the melting of the protein under extreme crowding. By deploying state-of-the art molecular simulations, supported by calorimetric experiments, we assess the role of the environment flexibility and of intermolecular electrostatic interactions. In particular, we show that the temperature-dependent flexibility of the macromolecular crowders, along with specific interactions, significantly alleviate the stabilizing contributions of the static volume effect.

## Introduction

Proteins work in a crowded space<sup>1</sup>. In fact, depending on the location, up to 30% by volume of the cell interior is occupied by various types of macromolecules, such as proteins, DNA, and RNA. This crowded environment affects both the mobility and the stability of proteins, but the finest details of this perturbation remain elusive. Indeed, an ideal excluded volume scenario predicts a stabilization effect due to the reduced available space for the extended unfolded conformations<sup>2,3</sup>. Experimental investigations report a far more complex situation whereby, depending on the crowding conditions, intermolecular interactions weaken the excluded volume contribution, and could lead to destabilization<sup>4-7</sup>.

For instance, NMR experiments at ambient temperature show that the B1 domain of protein G (GB1) is destabilized by homogenous crowders such as lysozyme and bovine serum albumin, but gains stability in a reconstituted cytosol<sup>7</sup>. On the contrary, the protein chymotrypsin inhibitor 2 (CI2) is always destabilized under similar conditions<sup>5,6</sup>. In-cell NMR measurements of the monomeric lambda repressor  $\lambda_{6-85}$  assessed unperturbed stability in the cytoplasm with re-

spect to a dilute condition *in vitro*<sup>4</sup>, while fluorescence microscopy experiments reports *in vivo* destabilization of the cellular retinoic acid binding protein (CRABP)<sup>8</sup>. Recently, fast relaxation imaging (FReI) experiments show that the destabilizing effect of the crowded environment on a variant of superoxide dismutase (SOD1) can be reverted by a single-point mutation<sup>9</sup>. Altogether, these findings indicate a critical modulation of the folding/unfolding free energy landscape by the complex cellular environment, including the presence of small osmolytes and ions. Several model systems have been set up to address these aspects including self-crowding<sup>10–13</sup>. The situation is even more intricate since the perturbation of the macromolecular crowding in cells depends on the compartment where the target protein is localized<sup>14,15</sup>. It is important to stress that, depending on the thermal response of the system, encoded for instance in the specific heat of unfolding, a crossover between stabilization and destabilization can occur by varying temperature<sup>16,17</sup>.

As the experimental essays face difficulties in probing the details of *in vivo* folding/unfolding, a natural support may come from computer simulations<sup>18</sup>. Unfortunately also the *in silico modeling* of realistic crowding is still a challenge due to the spread of length- and timescales involved<sup>19</sup>. Therefore, some theoretical investigations have formally considered the excluded volume effect on protein stability using simplified models<sup>3,16,20,21</sup>. In order to provide a more realistic vision, McGuffee and Elcock have reported a landmark study of a large system modelling the *E. coli* cytoplasm, utilising implicit solvent coarse-grained simulations<sup>22</sup>. The dynamics of the system was used to sample configurational states of the macromolecular packing. These states were then tested against the particle-insertion method<sup>23</sup> to estimate the free energy of unfolding of selected proteins. The calculations clearly shows that only when specific interactions are included, do the data for the test proteins approach the experimental measurement; weak stabilization for  $\lambda_{6-85}$  and destabilization for CRABP. In the same spirit but using an expensive all-atom resolution, a simulation of a model of *E. coli*<sup>24</sup> reports the destabilization of selected proteins monitored on a relatively short time scale (30 ns). Destabilization driven

by specific interactions was also computed in all atom simulations of smaller model crowded systems<sup>25,26</sup>. Recently, a multi-scale approach based on coarse-grained and atomistic simulations has been pioneered to study the thermal stability of target proteins in different crowding conditions and reproducing the experimental results, weak destabilization of SOD1 in bovine serum albumin (BSA)<sup>27</sup>, and existence of a crossover temperature for CI2 in lysozyme<sup>17</sup>.

Inspired by the analysis of neutron scattering (NS) experiments that we recently performed<sup>28</sup>, we examined the thermal stability of the chicken egg white lysozyme (CEWL) when placed under the extreme crowding condition of a solvated powder state, where it experiences an excluded volume of about 60%, a value much higher than that typical of the intracellular packing, see Fig. 1.A. Surprisingly, calorimetric experiments reported that the melting temperature of lysozyme in the fully hydrated powder state<sup>29,30</sup> is quite similar to that of dilute aqueous solutions<sup>31</sup>, and pointed out the limit of the excluded volume picture. However, when in the powder-like state water is replaced by glycerol, a dramatic stabilization effect is observed<sup>32</sup>. This shift is associated to an increase of the protein rigidity caused by the specificity of the hydrogen bond connectivity and viscosity of the solvent<sup>28,33-35</sup>. Therefore, solvated protein powders are appealing model systems to dissect the effects of the excluded volume, of the specific protein-protein interactions, and of solvation, and thus to clarify some aspects that are ubiquitous under the crowding condition beyond the specific molecularity of the system. Even if such model systems may appear not realistic with respect to the cellular environment, we recall that hydrated protein powders have been already used in the past to investigate and shed light on crucial biophysical protein properties, e.g. the dynamical transition<sup>36</sup>, the relationship among hydration and functionality<sup>37</sup>, and the link among vibrational motion and melting<sup>28</sup>. Moreover, our approach may give a significant contribution also in the pharmaceutical field, where therapeutic proteins are often stabilised in solid-state-like sugar- or polyol-based environments<sup>38</sup>.

Here, we have performed enhanced sampling molecular simulations to assess the thermal

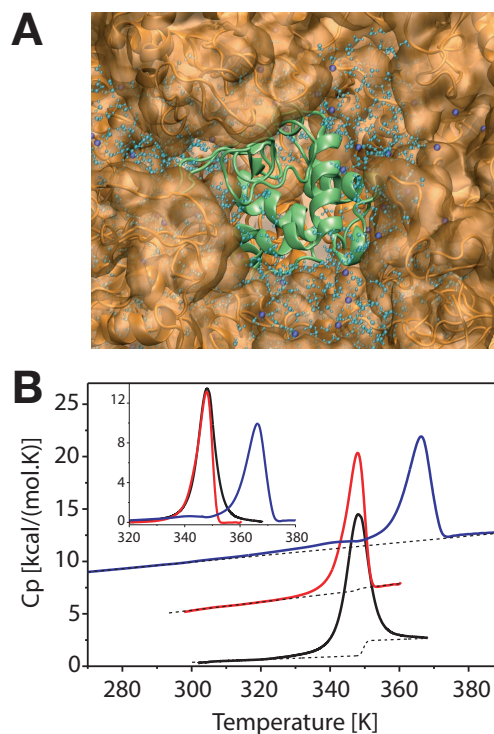


Figure 1: **Lysozyme in a solvated powder.** Top panel. Pictorial representation of a solvated powder of lysozyme proteins. A target protein (green lysozyme represented with the secondary structures) experiences the crowding effect of the surrounding proteins (orange lysozyme proteins represented with their secondary structures and via their external surface). The solvent molecules (blue water) percolate the interstitial space of the powder. Bottom panel. Specific heat of unfolding measured for the lysozyme protein in dilute water solution (black line), in the hydrated powder  $h=0.4$  (red line), and in a powder solvated with glycerol (blue line). In the main graph the baselines of the curves are explicitly represented (dashed lines) so to show the change of  $C_p$  upon unfolding. The experimental setup is described in the section Method and SI.

stability of the lysozyme protein in a powder state solvated either by water or glycerol. In addition, we also compare these systems to the reference dilute condition. To overcome the possible experimental ambiguities found in the literature concerning the investigated systems, we performed in-house calorimetry measurements to support the results from simulations and to allow consistent comparison of crowding and solvation effects for protein powders.

In order to estimate the contribution of excluded volume and environment plasticity, a set of virtual experiments were conducted by modifying the simulation conditions. Our results clearly show that the temperature-dependent flexibility of the crowding environment, in conjunction with protein-protein electrostatic interactions, dramatically reduce the effect of the extreme excluded volume of the powder state, in agreement with calorimetric experiments. The results obtained in this work for the powder systems complement the recent results concerning binary crowded solutions (CI2 in BSA and lysozyme) where the stabilising / destabilising contributions from excluded volume and quinary interactions were quantified via the same molecular simulation approach as a function of temperature<sup>17,27</sup>.

## Methods

### Molecular Dynamics Simulations

Molecular Dynamics simulations were performed on a lysozyme protein in dilute water solution, and on several lysozyme powder systems solvated in water and in glycerol. The system setups match exactly the experimental samples described in Ref.<sup>28</sup>. The simulations were performed with the CHARMM22/CMAP force-field for proteins<sup>39</sup>, CHARMM36 for the glycerol<sup>40</sup> and TIP3P CHARMM for water. We exploited a Hamiltonian replica exchange method with solute scaling (REST2) method<sup>41,42</sup> to enhance the sampling of the unfolding process by using 24 replicas. For each replica, the simulation was extended up to 500 ns. We achieved good replica energy overlap and high exchange rate (> 30%) in all the REST2 calculations. The details of

the system set-ups and simulations protocol are provided in SI.

## Differential Scanning Calorimetry

Calorimetry Experiments have been done on solid-state samples lysozyme+glycerol (1:1 in weight) and hydrated lysozyme (from 0.2 to 9 g water/g dry protein) by using the Perkin-Elmer Pyris Diamond Differential Scanning Calorimeter, while the Nano-DSC III Differential Scanning Calorimeter (TA instruments) has been used for lysozyme in water solution (1 mg/ml, pH=6). For further details see SI.

## Results

### Thermal stability in crowded powder systems

The thermal stability of a lysozyme protein in dilute aqueous solution and embedded in a solvated powder (with water or glycerol) was assessed by performing enhanced sampling MD simulations based on the REST2 technique<sup>41,42</sup>. In the powder systems, the enhanced sampling was performed by scaling the potential energy (which is equivalent to a thermal excitation at an effective temperature<sup>42</sup>) of only one of the protein molecules forming the powder, while the remainder ones, along with the solvent, evolved at unperturbed conditions in a reference bath temperature,  $T_{bath}$ . For these large systems, this strategy is a necessary short-cut in order to make the sampling computationally affordable by avoiding too many excited degrees of freedom, and thus a large number of replicas. Each replica is then associated to an effective temperature by a mean-field approach<sup>42</sup>.

A first set of calculations was performed considering  $T_{bath} = 300 \text{ K}$ <sup>28</sup>. By using the carbon- $\alpha$  root mean square displacement ( $C_\alpha$ -RMSD) with respect to the lysozyme crystallographic structure, and fixing a threshold value of 4.0 Å to distinguish folded and unfolded states<sup>28</sup>, we determined the fraction of folded and unfolded conformations sampled by the lysozyme as a



function of temperature<sup>42</sup>. The obtained protein melting curves are reported in Fig. 2.A. For powders, the calculated melting temperatures show an increase  $\Delta T_m^{sim} = T_{m,glyc}^{sim} - T_{m,water}^{sim}$  of  $\sim 70$  K on passing from water to glycerol, thus qualitatively reproducing the experimental trend where the stabilizing action of glycerol produces a  $\Delta T_m^{exp} = T_{m,glyc}^{exp} - T_{m,water}^{exp}$  of  $\sim 20$ -30 K, see Table 1 and Refs.<sup>29,32</sup>. However, each system's  $T_m^{sim}$ , and the shift of the melting  $\Delta T_m^{sim}$ , are much higher than the experimental values. In particular, the lysozyme in the hydrated powder is much more stable than in the dilute aqueous solution. On the contrary, when the environment is a pure aqueous solution as the lysozyme in the dilute condition, the melting temperature obtained from simulation  $T_{m,sol}^{sim} = 355K$  is very close to the experimental one (see Table 1 and Ref.<sup>31,43</sup>), as already observed for other proteins<sup>42,44</sup>.

These results indicate that the protein environment, including both the solvent molecules and the surrounding proteins, does play a key role to determine the thermal stability. Particularly, as we mentioned above, in the calculations of the powder systems, only one protein is affected by the thermal excitation while the rest of the environment evolves at the reference temperature  $T_{bath} = 300$  K. Therefore, in this system, the bath temperature  $T_{bath} = 300$  K does not allow the surrounding crowding proteins to perform the conformational jumps that actually take place upon heating. The effect of this sort of *frozen* environment is similar to what is observed in solids upon surface coating that shifts melting at higher temperatures<sup>45</sup>.

In order to prove the impact of the bath reference temperature, and therefore of the environment thermal response, we have performed a new set of simulations for the powder systems with  $T_{bath} = 350$  K, a temperature close to the melting of the lysozyme in dilute aqueous solutions. The extracted stability curves are reported in Fig. 2.B, and show striking features. Firstly, the melting temperatures of the powder systems downshift to values closer to the experiments. Secondly, the protein melting temperature in the hydrated powder approaches that for the protein in the dilute solution, with a relative shift of  $\Delta T_m^{sim} \simeq 25$  K, being more in line with the experiments (Table 1). These findings suggest that the weak perturbation of the

lysozyme thermal stability under the extreme crowding of the hydrated powder state relates to the temperature-dependent flexibility of the crowder agents. Clearly, the environment flexibility is controlled by the solvent as well, e.g. passing from water to the glycerol the environment gets rigidified<sup>34,35,46</sup>, and the resulting lysozyme melting temperature is higher than in the hydrated powder. We will discuss this aspect later on.

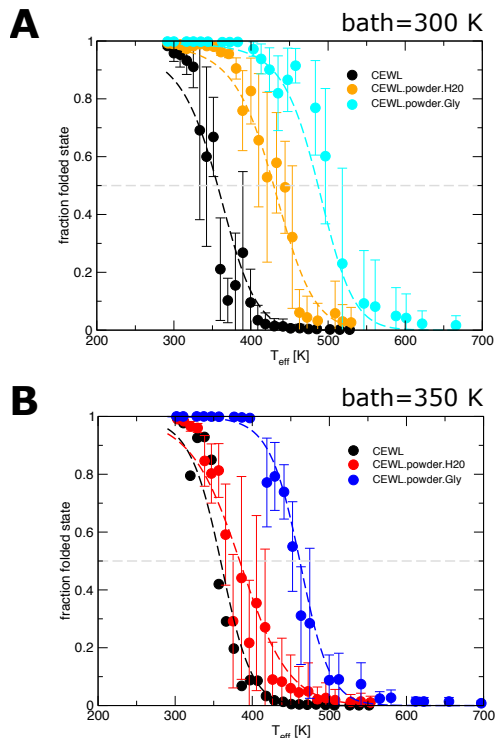


Figure 2: **Lysozyme stability curve.** Fraction of folded and unfolded states for a lysozyme protein in dilute condition (black circles), in a hydrated powder (orange/red circles), and in a powder solvated with glycerol (cyan/blue circles). The stability curves are obtained from REST2 simulations where the reference temperature of the protein crowders and the solvent is set to 300 K (Panel A) and 350 K (Panel B). The dashed lines correspond to a sigmoid fit to guide the eye.

## Dissecting the crowding and electrostatic contributions

An important piece of information on the role played by the molecular environment in affecting the protein thermal stability can be obtained by considering separately the effect of electrostatic

interactions, considered the essential source for destabilization under crowding<sup>6,7,22</sup>, from that of the excluded volume. To this purpose, we compare the thermal stability of CEWL in solution and in hydrated powder with that of a virtual hydrated powder system where the biomolecules acting as crowders were depleted of electrostatic intra- and intermolecular interactions (their charges were set to zero). In this way, the target lysozyme protein experiences an inert crowded environment interacting only via short-range Van der Waals potentials. The associated stability curves for the melting of the target lysozyme are reported in Fig. 3 (CEWL<sup>VdW</sup>.powder.H2O). At both bath temperatures, the extracted melting of the lysozyme in CEWL<sup>VdW</sup>.powder.H2O is  $T_m^{sim} \simeq 390$  K. However, when considering the rigid environment ( $T_{bath} = 300$  K) the excluded volume effect accounts for half of the stabilization, and the intermolecular electrostatic interactions add up providing extra-stabilization. The scenario is completely different when the thermal flexibility of the environment is accounted for ( $T_{bath} = 350$  K). In this case, while the melting temperature is the same as that of the system with full electrostatics, see Table 1, in a range of temperatures up to the melting, the target protein is more stable under the inert crowding than in the standard powder. This result confirms the idea explored in experimental and theoretical studies that specific interactions between proteins under crowding alleviate the excluded volume stabilising effect<sup>6,7,9,17,22,25-27</sup>.

## Flexibility of the crowders

We now examine how the dependence of the melting temperature upon the changes of the powder solvent, bath temperature, and electrostatic interactions is related to the crowders flexibility. Indeed, when thermalizing the protein crowders at a temperature close to the lysozyme melting,  $T_{bath} = 350$  K, it is expected that the flexibility of the crowders increases in a such a way that they do not behave as static obstructing objects. Similarly, when removing the intramolecular electrostatic interactions, the molecular mechanics of the force field is unbalanced, and the crowder proteins are expected to lose their intramolecular structural rigidity.

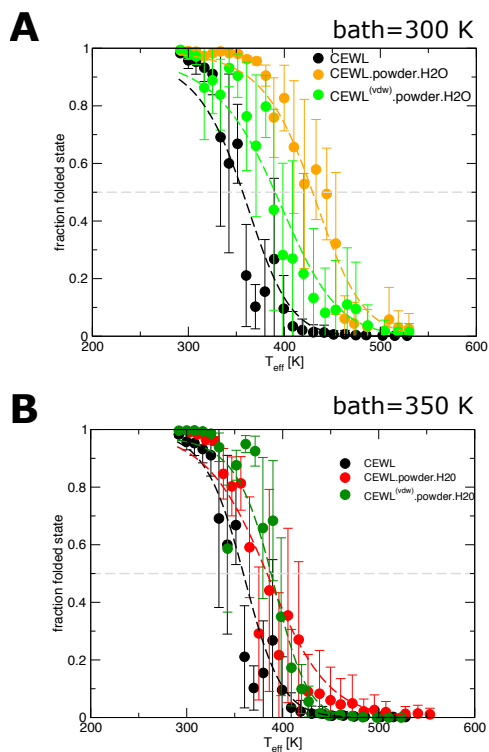


Figure 3: **Effect of inert crowders.** Fraction of folded and unfolded states for a lysozyme protein in dilute condition (black circles), in a hydrated powder (orange/red circles), and in a hydrated powder where the crowder proteins are modelled as apolar molecules (light and dark green circles). The stability curves are obtained from REST2 simulations where the reference temperature of the protein crowders and the solvent is set to 300 K (Panel A) and 350 K. (Panel B). The dashed lines correspond to a sigmoid fit to guide the eye.

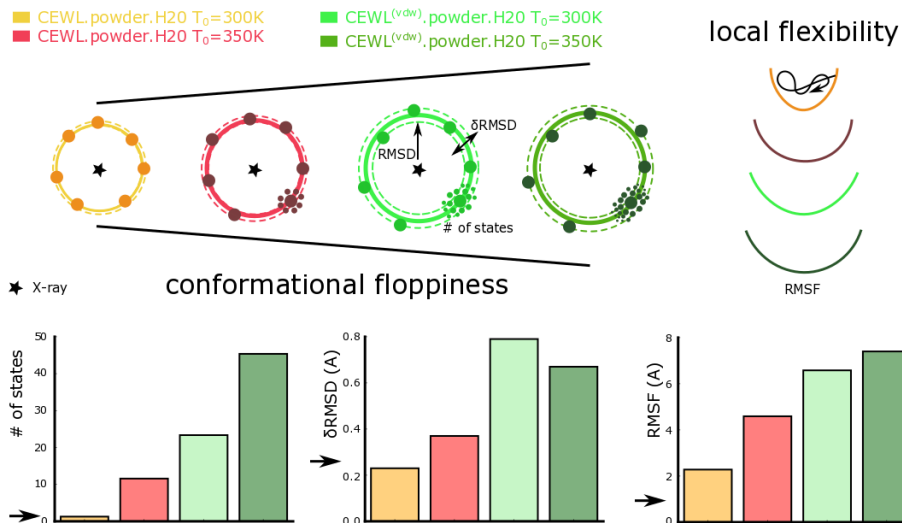


Figure 4: **Environment flexibility.** The floppiness of the crowding environment is quantified by different observables measuring local and global conformational flexibilities. In the bottom part of the figure we report in histograms the values of the average number of states obtained by conformational cluster analysis, the average fluctuations of the RMSD with respect to the X-ray lysozyme structure, and the RMSF. The average is carried out over the seven protein chains acting as crowders in the REST2 calculation. For the number of clusters, for each chain, the data are calculated from the first four replicas where the target lysozyme in the REST2 calculation is found folded. RMSD and RMSF are calculated for the replica corresponding to ambient temperature. The horizontal arrows on the side of the histograms indicate the average values obtained for the powder solvated with glycerol at  $T_{bath} = 350 K$ . In the upper part of the graph, the histogram data are pictorially represented with a simplified scheme. For each system, the circle radius is proportional to the value of the RMSD with respect to the X-ray structure, the inner and outer circle (dashed lines) indicated the effect of RMSD fluctuations. The filled small circles distributed along the main circumference represent the seven crowder chains. For sake of clarity for only one crowding chain we represent the ensemble of conformational states sampled during the simulations as a cloud of small filled circles.

This working hypothesis is here extensively probed by computing observables with the aim to monitor the crowder global and local flexibility, as shown in Fig. 4. We first focus on the global flexibility by counting the conformational states visited by each of the crowder proteins during the simulation. The sampled conformational states are distinguished by performing a cluster analysis based on the  $C_\alpha$ -RMSD with a cut-off of 2.5 Å. The average number of states visited by the seven crowder chains is reported in form of a bar plot in the Fig. 4, see also Table S1-S6. We clearly observe that for the system thermalized at  $T_{bath} = 300$  K, the crowders are substantially *frozen* in a given conformational state, while when the environment temperature is set to  $T_{bath} = 350$  K, or when their intramolecular interactions are altered (CEWL<sup>VdW</sup>.powder.H2O), they start to visit different conformations. The different degrees of flexibility between the systems are also reflected in the fluctuations of the RMSD with respect to the crystal structure ( $\delta$ RMSD), and the mean local fluctuations (RMSF), as we show in Fig. 4. As already discussed in the previous section, the melting temperature of lysozyme in the inert powder state is very similar at both the considered bath temperatures. This indicates that, once a flexible threshold is attained, the excluded volume contribution is no longer tunable. In the figure we also represent with black arrows the values for the number of clusters,  $\delta$ RMSD, and RMSF extracted for the powders solvated in glycerol at  $T_{bath} = 350$  K, see Table S5-S6 and Fig. S1. It is clear from the data that the glycerol solvent dramatically damps the local fluctuations (RMSF) and rigidifies the powder environment, and that even at high bath temperature  $T_{bath}$ , the sampled number of protein conformations is very small, see SI Table S3-S4. The aforementioned results are consistent with the picture emerging from neutron scattering investigations on the effect of crowding on the protein internal dynamics. In very highly concentrated solutions of bovine serum albumin protein, self-crowding has been shown to significantly reduce the rate of the diffusive local dynamics of backbone and side-chains<sup>47</sup>. On the other hand, the presence of osmolytes such as TMAO which are able to mediate soft interactions, may provide a decreased protein flexibility and an improvement in protein stability<sup>11</sup>. As for the effect of the glassy

molecular environment surrounding the protein surface, the behaviour of both the mean square displacements and the sub-picosecond timescale relaxation times of proteins in the presence of glycerol point towards a slowed-down and hindered internal biomolecule dynamics<sup>46,48</sup>.

## Excluded volume and quinary interactions

We have anticipated in the introduction that the melting temperature of the lysozyme protein in the powder state is almost identical -or slightly smaller- to that of the dilute solution<sup>11,29-31</sup>. In Fig. 5 we report the  $T_m$ s measured in a variety of experiments for lysozyme powder systems at different level of hydration, from dry state to fully hydrated. When the protein powder is dry the melting occurs at very high temperature ( $> 400 K$ ), but it decreases as hydration increases. Above the threshold  $h \simeq 0.4$ , the melting temperature no longer depends on the hydration level.

We have verified *in silico* that, once the thermal response of the environment is taken into account in the simulations, the lysozyme is only mildly thermally stabilised by the extreme crowding of the powder state compared to the dilute solution case. This is in agreement with the experimental results discussed above.

The crowders ability to accommodate the conformational changes of the unfolded state is therefore an essential aspect to include in the treatment of crowding effects on stability, especially for systems in which the target protein's and crowders' thermal responses are comparable. As already clearly pointed out by Zhou<sup>16</sup> the enthalpy/entropy compensation plays an essential role. In fact by using the experimental calorimetric data for the unfolding of lysozyme in pure water solution and in the hydrated powder (see Table 1), and the Gibbs-Helmholtz equation<sup>54</sup>, we have verified that, even if the measured melting temperatures  $T_m^{exp}$ s are very close when moving from solution to hydrated powder, the powder state provides a stabilization free energy of  $\Delta\Delta G_{f/u} = 1.5 kcal/mol$  at ambient temperature, see Fig. 6. By applying the Ideal Thermal Protein model introduced by Ghosh and Dill<sup>55</sup> (see SI), the stabilization due to the excluded

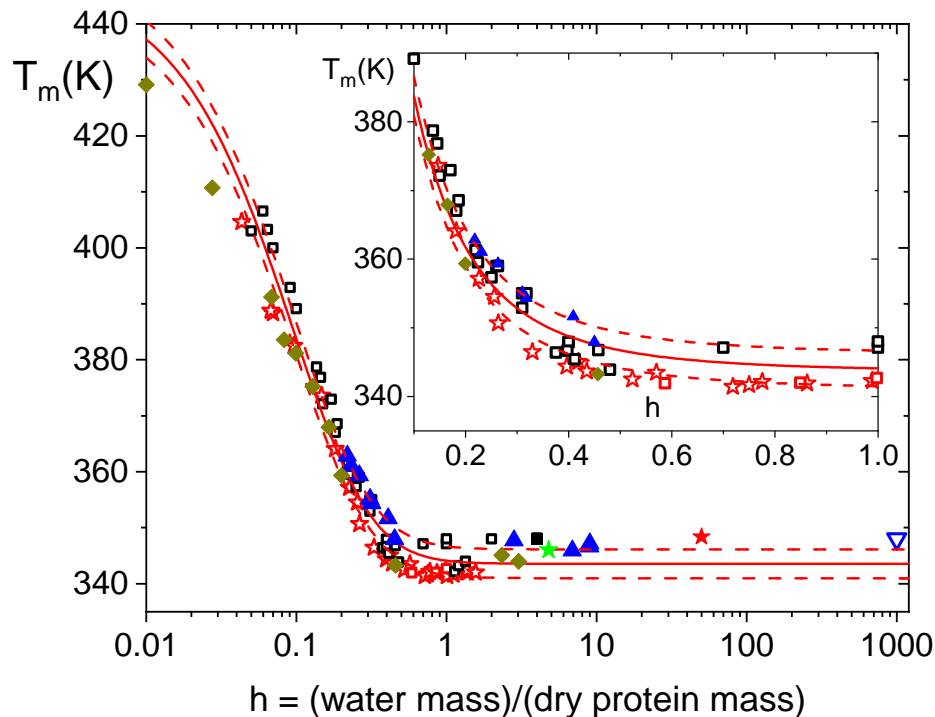


Figure 5: **Melting temperatures.** Peak melting from thermograms obtained versus hydration ratio  $h$  for a series of samples at different scan rates. Continuous and dashed lines are from global fitting of literature data<sup>49</sup>, average and confidence bands, respectively. Data obtained at scan rate 10 K/min are from this work (blue solid triangles), Ref.<sup>50</sup> (black open squares), Ref.<sup>51</sup> (red solid stars), Ref.<sup>52</sup> (black solid squares). Other data are from 5 K/min (gold solid diamonds)<sup>30</sup>, 2.5 K/min (red open stars)<sup>29</sup>, 1 K/min (green solid star)<sup>53</sup> and this work (blue open triangle). Inset show an enlargement for the range  $h=0.1-1.0$ .

volume in the powder is expected to be much larger,  $\sim 4 \text{ kcal/mol}$  at  $T = 300 \text{ K}$ , and would cause an increase in the melting temperature of about  $13 \text{ K}$  (red-dashed-line in Fig. 6). The experimental data therefore suggest that the pure excluded volume effect must be weakened by other contributions.

To further assess the role of environment flexibility, we have shown that moving from water to glycerol, the rigidity of the environment strengthens (see Fig. 4), and as a consequence, the thermal stability of lysozyme increases in agreement with experiments. This stabilization is associated with a larger change in the unfolding enthalpy in glycerol as compared to water in both experiments and simulations. From our experiments, it is difficult to provide a neat



**Table 1: Thermodynamic Parameters of Protein Stability.** The reported experimental data have been extracted from calorimetry experiments (see SI for details and a comparison with literature) . For the powder system in H2O we averaged the  $T_m$ s values obtained at  $h=0.42$  and  $0.45$ .

| <b>System</b> (experiment) | $T_m^{exp}$ [K] | $\Delta H_m^{exp}$ [kcal/mol] |
|----------------------------|-----------------|-------------------------------|
| CEWL                       | 348             | 114±5                         |
| CEWL.powder.H2O            | 349.5           | 89±2                          |
| CEWL.powder.Gly            | 366             | 96±2                          |

estimate of the specific heat of unfolding, but as indicated by others<sup>29,32</sup>, it is close to zero in the powder states. The stabilization in the powder state induced by the glycerol solvent when compared to water, is therefore mainly due to an upshift of the stability curve as visible in Fig. 6, and relates to the specific interactions of the solvent with the protein, e.g. hydrogen-bond connectivity.

In the REST2 scheme, the enhanced sampling is exact (in the statistical mechanical sense) at the bath reference temperature,  $T_{bath}$ , while it is approximated for other replicas whose effective temperature is assigned via a mean field formulation<sup>42</sup>. By analysing the simulations performed at  $T_{bath} = 350 K$ , we can quantify at this temperature the free energy contribution due to exclude volume effect, and the modulation stemming from the electrostatic interactions. From the curves reported in Fig. 3.B, the stability contribution is deduced by comparing the fraction of folded ( $[f]$ ) and unfolded states ( $1 - [f]$ ) for the different systems. For instance, in order to estimate the effect of inert excluded volume, we compared the CEWL<sup>VdW</sup> system ( $[f]_{VdW}$ ) with the dilute condition ( $[f]_0$ ), and obtained  $\Delta\Delta G_{f/u} = -k_bT(\ln([f]_{VdW}/(1 - [f]_{VdW})) - \ln([f]_0/(1 - [f]_0))) \simeq 1.7$  kcal/mol. On the contrary, for the CEWL hydrated powder system where the electrostatic interactions are included in the calculations, the crowding stabilization with respect to the dilute condition reduces by a factor of two,  $\Delta\Delta G_{f/u} \simeq 0.9$  kcal/mol. Our finding is consistent with the idea that repulsive protein/protein interactions are prone to counterbalance the excluded volume stabilising effect<sup>6,7</sup>, and with the fact that each lysozyme molecule in the powder carries a net positive charge.

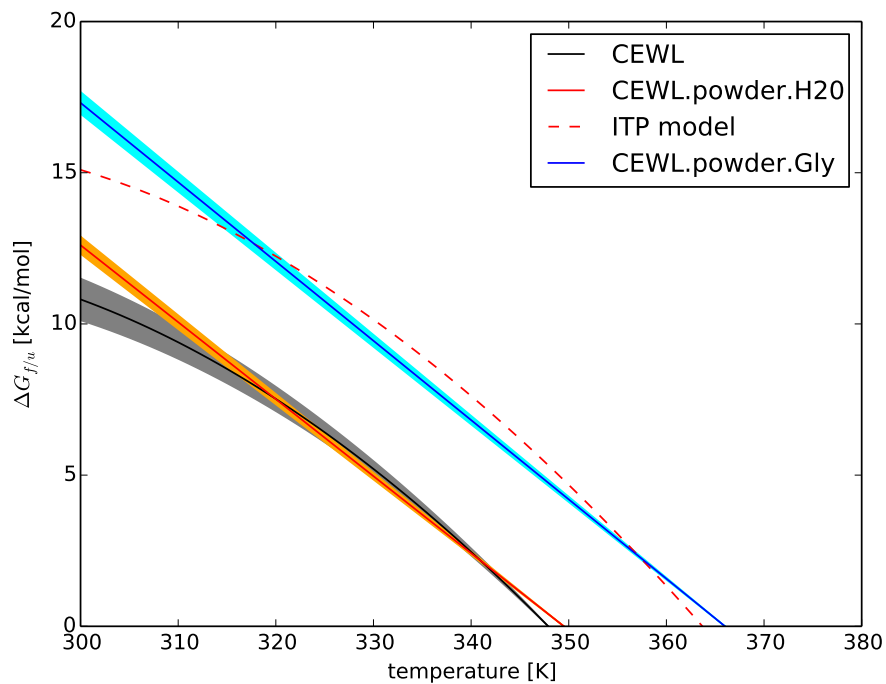


Figure 6: **Stability curves.** Stability curves reconstructed using the Gibbs-Helmoltz equation<sup>54</sup>. The thermodynamic parameters for the thermal unfolding of lysozyme in dilute solution (black line), in the hydrated powder (red line) and in the powder solvated with glycerol (blue line) are extracted from our calorimetry experiments. The shadow region in each curve indicates the uncertainty associated to the  $\Delta H_m$ 's error. The stability curve deduced from the Ideal Thermal Protein model<sup>55</sup>, see below, to account for the excluded volume in the hydrated lysozyme protein is also reported (dashed red line).

Notably, by moving from standard to inert crowding, the number of contacts between the target lysozyme and the crowder proteins increases of about 11%, and correspondingly, the number of contacts with the water molecules decreases. As a net effect, the crowdors become more sticky and the lubrication action of the hydration layer is reduced. Similar stabilizing effects are observed e.g. for protein dimers that are more thermally stable as compared to the isolated monomers<sup>56,57</sup>, but they are to contrast with the effect of denaturing osmolytes, that are in fact widely accepted to accumulate at the protein interface and lead to protein unfolding<sup>58</sup>. In fact, the size of the crowder is of special importance, with a large sticky crowder hindering protein unfolding, while small crowdors can accumulate at the protein interface and

then penetrate into its structure to unfold it.

## Conclusions

In this work, we have inquired how the crowded molecular environment of a powder state affects the stability of the lysozyme protein. We have performed advanced *in silico* experiments in order to assess the thermal unfolding of lysozyme as well as to dissect the role of crowding rigidity on the excluded volume effect, and to inspect the role of electrostatic intermolecular interactions and solvation. We have shown that protein stability can be modulated by the flexibility of the crowding agents and specific interactions between the protein and its surrounding environment. The results from this and other investigations<sup>17,24,27</sup> show that the present computational *savoir-faire* is mature to provide systematic insights on the crowding effect on protein thermal stability in cell-like conditions and support experiments<sup>5-7,9,15,16</sup>. Namely, the REST2 approach has been proved to be a powerful tool to study thermal unfolding under crowding where the system size discourages the use of other techniques (e.g. standard REMD). For example, it was recently applied to quantify the contribution of quinary interactions on the destabilisation of the SOD1 protein in a crowded BSA solution<sup>9,27</sup>, and to estimate the delicate balance among quinary interactions and excluded volume contributions acting on the stability of the CI2 protein under different crowding conditions (BSA vs lysozyme)<sup>17</sup>. In the present study we have shown that the environment flexibility controlled by its thermal response modulates the crowding excluded volume effect, complementing our finding in previous studies. Therefore, the thermal response of the bath can be taken into account in the REST2 simulations of crowded solutions when the stability of the crowders and of the target protein are comparable. For instance, we are exploring a protocol where a series of independent REST2 simulations with different reference temperatures are run in parallel; alternatively it is possible to generate solution states where the crowders are either placed in folded or unfolded conformations so as to mimic the extreme two-state environmental conditions.

The investigation of the role of the solvent, metabolites, as well as the fluctuations of the crowding environment in vivo<sup>59</sup>, represent future research prospects. Our approach may give a significant contribution also in the pharmaceutical field, where therapeutic proteins are often stabilised in solid-state-like sugar- or polyol-based environments

## Supporting Information

Supporting data on calorimetry experiments, excluded volume model, observables relating to protein crowders flexibility.

## Acknowledgement

FS acknowledge funding from the ERC (FP7/2007-2013) Grant Agreement no.258748 and supports from "Initiative d'Excellence" program from the French State (Grant "DYNAMO", ANR-11-LABX-0011-01). Part of his work was performed using HPC resources from GENCI (Grants x2016(7)076818).

## References

- (1) Ellis, R. J.; Minton, A. P. Cell Biology: Join the Crowd. *Nature* **2003**, *4*, 27–28.
- (2) Zhou, H.-X.; Rivas, G.; Minton, A. P. Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. *Ann. Rev. Biophys.* **2008**, *37*, 375–397.
- (3) Zhou, H.-X. Protein Folding in Confined and Crowded Environments. *Arch. Biochem. Biophys.* **2008**, *469*, 76–82.

- (4) Ghaemmaghami, S.; Oas, T. Quantitative Protein Stability Measurement in Vivo. *Nat. Struct. Mol. Biol.* **2001**, *8*, 879–882.
- (5) Miklos, A. C.; Sarkar, M.; Wang, Y.; Pielak, G. J. Protein Crowding Tunes Protein Stability. *J. Am. Chem. Soc.* **2011**, *133*, 7116–7120.
- (6) Sarkar, M.; Smith, A. E.; Pielak, G. J. Impact of Reconstituted Cytosol on Protein Stability. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 19342–19347.
- (7) Monteith, W. B.; Pielak, G. J. Residue Level Quantification of Protein Stability in Living Cells. *Proc. Natl. Acad. Sci. USA* **2014**, *31*, 11335–11340.
- (8) Ignatova, Z.; Krishnan, B.; Bombardier, J. P.; Marcelino, A. M. C.; Hong, J.; Gierasch, L. M. From the Test Tube to the Cell: Exploring the Folding and Aggregation of a b-Clam Protein. *Biopolymers* **2007**, *88*, 157–163.
- (9) Gnutt, D.; Timr, S.; Ahlers, J.; König, B.; Manderfeld, E.; Heyden, M.; Sterpone, F.; Ebbinghaus, S. Stability Effect of Quinary Interactions Reversed by Single Point Mutations. *J. Am. Chem. Soc.* **2019**, *141*, 4660–4669.
- (10) Erlkamp, M.; Grobelny, S.; Winter, R. Crowding Effects on the Temperature and Pressure Dependent Structure, Stability and Folding Kinetics of Staphylococcal Nuclease. *Phys. Chem. Chem. Phys.* **2014**, *16*, 5965–5976.
- (11) Al-Ayoubi, S. R.; Schummel, P. H.; M. Golub, M.; Peters, J.; Winter, R. Influence of Cosolvents, Self-Crowding, Temperature and Pressure on the sub-Nanosecond Dynamics and Folding Stability of Lysozyme. *Phys. Chem. Chem. Phys.* **2017**, *19*, 14230–14237.
- (12) Sörensen, T.; Leeb, S.; Danielsson, J.; Oliveberg, M. Polyanions Cause Protein Destabilization Similar to That in Live Cells. *Biochemistry* **2021**, *60*, 735–746.

- (13) X.Song,; L.An,; M.Wang,; J.Chen,; Z.Liu,; Yao, L. Osmolytes Can Destabilize Proteins in Cells by Modulating Electrostatics and Quinary Interactions. *ACS Chem. Bio.* **2021**, <https://doi.org/10.1021/acscchembio.1c00024>.
- (14) Ebbinghaus, S.; Dhar, A.; McDonald, J. D.; Gruebele, M. Protein Folding Stability and Dynamics Imaged in a Living Cell. *Nature Methods* **2010**, *7*, 319–323.
- (15) Dhar, A.; Girdhar, K.; Singh, D.; Gelman, H.; Ebbinghaus, S.; Gruebele, M. Protein Stability and Folding Kinetics in the Nucleus and Endoplasmic Reticulum of Eucaryotic Cells. *Biophys. J.* **2011**, *101*, 421–430.
- (16) Zhou, H.-X. Polymer Crowders and Protein Crowders Act Similarly on Protein Folding Stability. *Febs Lett.* **2013**, *587*, 394–397.
- (17) Timr, S.; Sterpone, F. Stabilizing or Destabilizing: Simulations of Chymotrypsin Inhibitor 2 under Crowding Reveal Existence of a Crossover Temperature. *J. Phys. Chem. Lett.* **2021**, *12*, 1741–1746.
- (18) Gruebele, M.; Thirumalai, D. Perspective: Reaches of Chemical Physics in Biology. *J. Chem. Phys.* **2013**, *139*, 121701.
- (19) Frembgen-Kesner, T.; Elcock, A. H. Computer Simulations of the Bacterial Cytoplasm. *Biophys. Rev.* **2013**, *5*, 109–119.
- (20) Cheung, M. S.; Klimov, D.; Thirumalai, D. Molecular Crowding Enhances Native State Stability and Refolding Rates of Globular Proteins. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 4753–4758.
- (21) Mittal, J.; Best, R. Dependence of Protein Folding Stability and Dynamics on the Density and Composition of Macromolecular Crowders. *Biophys. J.* **2010**, *98*, 315–320.

- (22) McGuffee, S. R.; Elcock, A. H. Diffusion, Crowding and Protein Stability in a Dynamic Molecular Model of the Bacterial Cytoplasm. *PLoS Comput. Biol.* **2010**, *6*, e1000694.
- (23) Widom, B. Some Topics in the Theory of Fluids. *J. Chem. Phys.* **1963**, *39*, 2808–2812.
- (24) Yu, I.; Mori, T.; Ando, T.; Harada, R.; Jung, J.; Sugita, Y.; Feig, M. Biomolecular Interactions Modulate Macromolecular Structure and Dynamics in Atomistic Model of a Bacterial Cytoplasm. *eLife* **2016**, *5*, e19274.
- (25) Feig, M.; Sugita, Y. Variable Interactions between Protein Crowders and Biomolecular Solutes Are Important in Understanding Cellular Crowding. *J. Phys. Chem. B* **2012**, *116*, 599–605.
- (26) Harada, R.; Tochio, N.; Kigawa, T.; Sugita, Y.; Feig, M. Reduced Native State Stability in Crowded Cellular Environment Due to Protein-Protein Interactions. *J. Am. Chem. Soc.* **2013**, *135*, 3696–3701.
- (27) Timr, S.; Gnutt, D.; Ebbinghaus, S.; Sterpone, F. The Unfolding Journey of Superoxide Dismutase 1 Barrels Under Crowding: Atomistic Simulations Shed Light on Intermediate States and Their Interactions With Crowders. *J. Phys. Chem. Lett.* **2020**, *11*, 4206–4212.
- (28) Katava, M.; Stirnemann, G.; Zanatta, M.; Capaccioli, S.; Pachetti, M.; Ngai, K. L.; Sterpone, F.; Paciaroni, A. Critical Structural Fluctuations of Proteins Upon Thermal Unfolding Challenge the Lindemann Criterion. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 9361–9366.
- (29) Fujita, Y.; Noda, Y. Effect of Hydration on the Thermal Denaturation of Lysozyme as Measured by Differential Scanning Calorimetry. *Bull. Chem. Soc. Japan* **1978**, *51*, 1567–1568.

- (30) Bell, L. N.; Hageman, M. J.; Muraoka, L. M. Thermally Induced Denaturation of Lyophilized Bovine Somatotropin and Lysozyme As Impacted by Moisture and Excipients. *J. Pharm. Sci.* **1995**, *84*, 707–712.
- (31) Burova, T. V.; Grinberg, N. V.; Grinberg, V. Y. Calorimetric Evidence for a Native-like Conformation of Hen Egg-white Lysozyme Dissolved in Glycerol. *Biochimica et Biophysica Acta* **2000**, *1478*, 309–317.
- (32) Cinelli, S.; De Francesco, A.; Onori, G.; Paciaroni, A. Thermal Stability and Internal Dynamics of Lysozyme as Affected by Hydration. *Phys. Chem. Chem. Phys.* **2004**, *6*, 3591–3595.
- (33) Cornicchi, E.; Onori, G.; Paciaroni, A. Picosecond-time-scale Fluctuations of Proteins in Glassy Matrices: The Role of Viscosity. *Phys. Rev. Lett.* **2005**, *95*, 158104.
- (34) Dirama, T.; Carri, G.; Sokolov, A. Coupling Between Lysozyme and Glycerol Dynamics: Microscopic Insights from Molecular-Dynamics Simulations. *J Chem Phys* **2005**, *122*, 244910.
- (35) Ghattyvenkatakrishna, P.; Carri, G.; Sokolov, A. Effect of Glycerol-water Binary Mixtures on the Structure and Dynamics of Protein Solutions. *J Biomol Struct Dyn* **2014**, *32*, 424–437.
- (36) Doster, W.; Cusack, S.; Petry, W. Dynamical Transition of Myoglobin Revealed by Inelastic Neutron Scattering. *Nature* **1989**, *337*, 754–757.
- (37) Schinkel, J. E.; Downer, N. W.; Rupley, J. A. Hydrogen Exchange of Lysozyme Powders. Hydration Dependence of Internal Motions. *Biochemistry* **1985**, *24*, 352–366.
- (38) R. Nayar, M. C. M. *Rational Design of Stable Protein Formulations: Theory and Practice*; Plenum Pub Corp, 2002.



- (39) MacKerell, A. D.; Feig, M.; Brooks(III), C. L. Extending the Treatment of Backbone Energetics in Protein Force Fields: Limitations of Gas-phase Quantum Mechanics in Reproducing Protein Conformational Distributions in Molecular Dynamics Simulations. *J. Comput. Chem.* **2004**, *25*, 1400–1415.
- (40) Hatcher, E. R.; Guvench, O.; MacKerell, A. D. CHARMM Additive All-Atom Force Field for Acyclic Polyalcohols, Acyclic Carbohydrates, and Inositol. *J. Chem. Theory. Comput.* **2009**, *5*, 1315–1327.
- (41) Wang, L.; Friesner, R.; Berne, B. Replica Exchange with Solute Scaling: A More Efficient Version of Replica Exchange with Solute Tempering (REST2). *J. Phys. Chem. B* **2011**, *115*, 11305.
- (42) Stirnemann, G.; Sterpone, F. Recovering Protein Thermal Stability Using All-Atom Hamiltonian Replica-Exchange Simulations in Explicit Solvent. *J. Chem. Theory. Comput.* **2015**, *11*, 5573–5577.
- (43) Shih, P.; Holland, D. R.; Kirsch, J. F. Thermal Stability Determinants of Chicken Egg-White Lysozyme Core Mutants: Hydrophobicity, Packing Volume, and Conserved Buried Water Molecules. *Protein Sci.* **1995**, *4*, 2050–2062.
- (44) Stirnemann, G.; Sterpone, F. Mechanics of Protein Adaptation to High Temperatures. *J. Phys. Chem. Lett.* **2017**, *8*, 5884–5890.
- (45) Jin, Z.; Gumbusch, P.; Lu, K.; Ma, E. Melting Mechanisms at the Limit of Superheating. *Phys. Rev. Lett.* **2001**, *87*, 0557031–0557034.
- (46) Paciaroni, A.; Cinelli, S.; Onori, G. Effect of the Environment on the Protein Dynamical Transition: A Neutron Scattering Study. *Biophys. J.* **2002**, *83*, 1157–1164.

- (47) Grimaldo, M.; Roosen-Runge, F.; Hennig, M.; Zanini, F.; Zhang, F.; Jalarvo, N.; Zamponi, M.; Schreiber, F.; Seydel, T. Hierarchical Molecular Dynamics of Bovine Serum Albumin in Concentrated Aqueous Solution Below and Above Thermal Denaturation. *Phys. Chem. Chem. Phys.* **2015**, *17*, 4645–4655.
- (48) Paciaroni, A.; Orecchini, A.; Cinelli, S.; Onori, G.; Lechner, R. E.; Pieper, J. Protein Dynamics on the Picosecond Timescale as Affected by the Environment: a Quasielastic Neutron Scattering Study. *Chem. Phys.* **2003**, *292*, 397–404.
- (49) Martínez, L. M.; Videa, M.; Cruz-Angeles, J.; Rodríguez, S. L. Controlled Water Content for Evaluation of Denaturation Temperature of Freeze-dried Enzymes. *Thermochimica Acta* **2016**, *638*, 52–57.
- (50) Yazhen, F. Effect of Hydration on the Thermal Stability of Lysozyme Studies on Thermodynamic Regularity of Pre-thermodenaturation New Peak and Denaturation Peak. *Kexue Tongbao* **1985**, *30*, 1112–1117.
- (51) Fujita, Y.; Miyanaga, A.; Noda, Y. Effect of Alcohols on the Thermal Denaturation of Lysozyme as Measured by Differential Scanning Calorimetry. *Bull. Chem. Soc. Jpn.* **1979**, *53*, 3659–3662.
- (52) Martínez, L.; Videa, M.; Mesquita, J. Design, Construction and Calibration of a Portable Multi Sample DTA Setup. *Thermochim. Acta* **2013**, *560*, 89–94.
- (53) Hedoux, A.; Willart, J. F.; Ionov, R.; Affouard, F.; Guinet, Y.; Paccou, L.; Lerbret, A.; Descamps, M. Analysis of Sugar Bioprotective Mechanisms on the Thermal Denaturation of Lysozyme from Raman Scattering and Differential Scanning Calorimetry Investigations. *J. Phys. Chem. B* **2006**, *110*, 22886–22893.
- (54) Bechtel, W. J.; Schellman, J. A. Protein Stability Curves. *Biopolymers* **1987**, *26*, 1859–1877.

- (55) Gosh, K.; Dill, K. A. Computing Protein Stabilities from their Chain Lengths. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 10649–10654.
- (56) Loveridge, E. J.; Rodriguez, R. J.; Swanwick, R. S.; Allemann, R. K. Effect of Dimerization on the Stability and Catalytic Activity of Dihydrofolate Reductase from the Hyperthermophile *Thermotoga Maritima*. *Biochemistry* **2009**, *48*, 5922–5933.
- (57) Maffucci, I.; Laage, D.; Stirnemann, G.; Sterpone, F. Differences in Thermal Structural Changes and Melting between Mesophilic and Thermophilic Dihydrofolate Reductase Enzymes. *Phys. Chem. Chem. Phys.* **2020**, *22*, 18361–18373.
- (58) Canchi, D. R.; García, A. E. Cosolvent Effects on Protein Stability. *Annu Rev Phys Chem* **2013**, *64*, 273–93.
- (59) Dave, K.; Davtyan, A.; Papoian, G. A.; Gruebele, M.; Platkov, M. M. Environmental Fluctuations and Stochastic Resonance in Protein Folding. *Chem. Phys. Chem.* **2016**, *17*, 1341–1348.

# TOC Graphic

