Protein Folding

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Single-Molecule-Level Evidence for the Osmophobic Effect**

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Organic osmolytes are low-molecular-weight osmotically active compounds, which are ubiquitous in living systems and are able to modulate protein stability. Among them, those that act as folding agonists, enhancing the stability of the native structure of proteins, such as trimethylamine N-oxide, betaine, sarcosine, proline, trehalose, sucrose, glycerol, sorbitol, and dimethylsulphoxide (DMSO), are collectively called protecting osmolytes or "chemical chaperones".[1–3] One rather puzzling feature of these compounds is that they are able to affect the folding of very diverse proteins in similar ways, suggesting that they might act according to a general mechanism,[4–10] in contrast to the more specific mechanisms employed by chaperone proteins.[11,12] In fact, the most widely accepted theory to rationalize their mode of action proposes that the addition of a protecting osmolyte to water as a co-solvent results in diminished solvent quality for the protein backbone, thus making intra-peptide backbone–backbone hydrogen bonds energetically more favorable than those between the backbone and the solvent.[1]

This effect, known as the osmophobic effect, implies that protecting osmolytes have a universal, indirect mode of action, which does not entail the presence of any specific binding sites for the osmolyte on the protein in any of its states, including the folding/unfolding transition state. On the other hand, evidence of such a direct participation have been recently provided by experimental studies for specific protein–osmolyte combinations.[13–16]

Herein, we provide experimental evidence, at the single-molecule level, that the osmolyte DMSO protects the native state of a globular protein against mechanical unfolding without any active complexation of the osmolyte molecules into its unfolding transition state. Apart from slowing down the spontaneous unfolding rate of the protein, we show that the osmolyte also simultaneously accelerates its folding rate. The kinetic description of the observed stabilization mechanism strongly supports a backbone-based theory of the osmophobic effect.

We employed atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS)[19] to characterize the effect of DMSO on the folding and unfolding kinetics of a globular protein domain, namely the B1 immunoglobulin binding domain of protein G from streptococcus (herein referred to as GB1), which behaves as a two-state folding protein on AFM experimental timescales.[20–23]

SMFS mechanical unfolding and refolding[17] experiments were performed on polyprotein constructs made up of either eight or sixteen tandem repeats of the GB1 domain.[20] We used buffered solutions with five different concentrations of DMSO ranging from 0% to 50% v/v (see the Supporting Information, Section 1 for the detailed SMFS experimental methods, and Section 2 for preparation of protein constructs).

The unfolding experiments were performed using three independent and complementary SMFS modes of operation (Figure 1), which consistently led to the same result: in the presence of DMSO, the spontaneous unfolding rate of GB1 at zero applied force is negatively correlated with DMSO concentration, meaning that DMSO kinetically protects the folded state against unfolding. This protection manifests itself as an increase of the average mechanical unfolding forces at all loading rates in the velocity clamp SMFS mode (Figure 2a), and a decrease of the force-dependent unfolding rates in force ramp and force clamp experiments (Supporting Information, Section 4 and Figure S1). The refolding experiments were instead performed using a variable time lapse, double pulse procedure[17,24] and showed that DMSO increases the spontaneous folding rate of GB1 (please refer to Supporting Information, Section 5 for the experimental details on this procedure).

The data (Figure 2a) makes it possible to map the mechanical unfolding energy landscape of GB1 at each investigated DMSO concentration, extracting (as detailed elsewhere)[25] the two fundamental kinetic parameters of the

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Supporting information for this article, including details of velocity clamp SMFS experiments, polyprotein synthesis and purification, statistical analysis of the velocity clamp data, force ramp and force clamp SMFS experiments, and double-pulse refolding SMFS experiments, is available on the WWW under http://dx.doi.org/10.1002/anie.201006714.

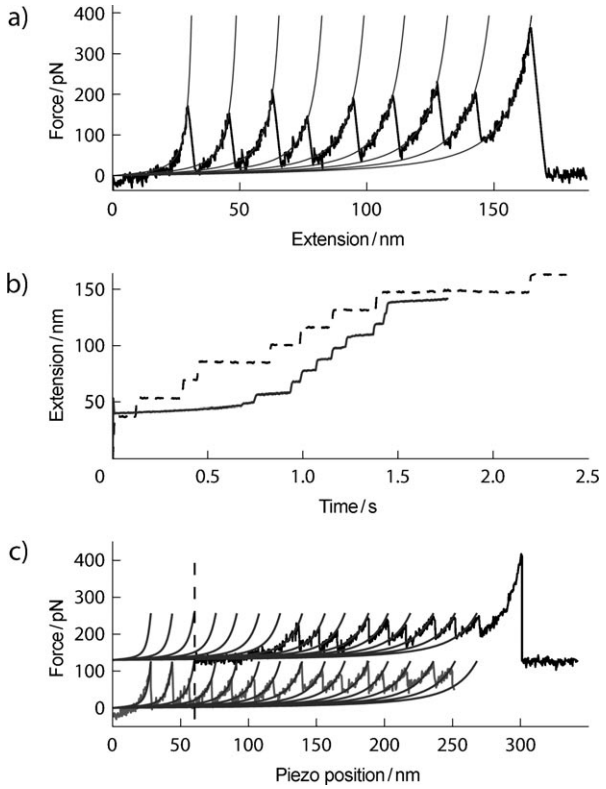

Figure 1. Representative examples of the type of data captured under different AFM modes of operation used in our study: protein unfolding under a) velocity-clamp, b) force-clamp (continuous line) and force-ramp (dashed line), and c) protein refolding using a custom double-pulse method[17] (described in detail in Supporting Information, Section 5). An exhaustive explanation of the specific advantages of each SMFS mode of operation can be found elsewhere.[18,19] Consistently observing the same phenomenon with all three modes (see main text) allowed us to rule out the possibility of observing experimental artifacts caused by the specifics of each method.

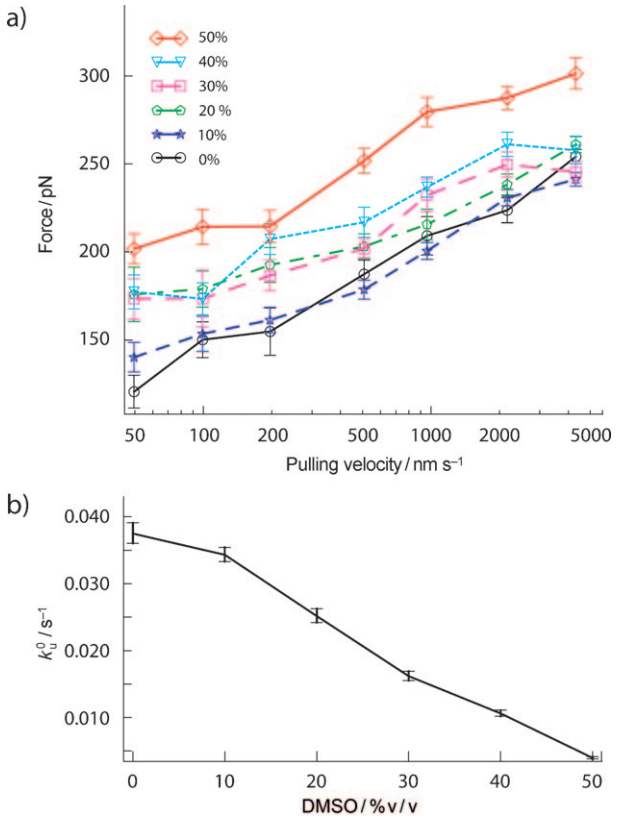

Figure 2. a) Average mechanical unfolding force by pulling velocity at various DMSO concentrations (values shown as % v/v). The error bars extend two standard errors of the mean unfolding force above and below the mean value. (The number of unfolding events are given in the Supporting Information, Table S1.) b) Spontaneous dissociation rate and 68.3 % confidence intervals with varying DMSO concentration, calculated with a fixed Δx_u value of 0.165 nm.

widely employed Kramer two-state model:[26] the distance between the native state and the transition state along the reaction coordinate Δx_u (Figure 3) and the spontaneous unfolding rate in absence of an applied force k_u^0 (see the Supporting Information, Section 3 for the data analysis methods). Importantly, as the reaction coordinate coincides with the direction of elongation of the protein in SMFS mechanical unfolding experiments, the extracted value of Δx_u is highly sensitive to changes in the geometry of the transition state.[13,14] The other parameter of interest, namely k_u^0, is exponentially dependent on the height of the unfolding transition barrier ΔG_u according to the formula $k_u^0 \propto \exp[-\Delta G_u/(K_b T)]$. Therefore, the difference in the heights of the unfolding transition barrier ($\Delta\Delta G_u$) in two different conditions (for example, DMSO concentrations) can be computed from the logarithm of the ratio of the k_u^0 values in the two conditions.

The obtained Δx_u values at each DMSO concentration are in very good accord with previously published Δx_u values of GB1 in absence of DMSO.[20] More specifically, the Δx_u values remained almost unchanged around a mean value of 0.165 nm, without any apparent trend (see Supporting

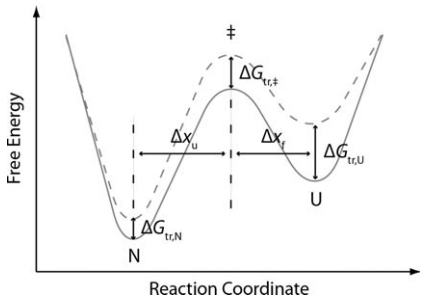

Figure 3. Representation of the unfolding–refolding energy landscape of a two-state protein under the effect of a protecting osmolyte (—— water only, ----- water + protecting osmolyte). Kinetically, a protecting osmolyte accelerates protein folding by increasing the height of the unfolding activation barrier ($\Delta\Delta G_U = \Delta G_{tr,\ddagger} - \Delta G_{tr,N} > 0$) and decreasing the height of the folding activation barrier ($\Delta\Delta G_f = \Delta G_{tr,U} - \Delta G_{tr,\ddagger} > 0$), where $\Delta G_{tr,\ddagger}$, $\Delta G_{tr,N}$, and $\Delta G_{tr,U}$ represent the free energy of transfer from water to the water–osmolyte mixture of the transition (‡), native (N), and unfolded (U) state, respectively. It follows as a thermodynamical consequence that $\Delta\Delta G = \Delta G_{tr,U} - \Delta G_{tr,\ddagger} = \Delta\Delta G_f + \Delta\Delta G_u > 0$, where ΔG is the free energy difference between the unfolded and native states, therefore generating the inequality commonly referred to as the osmophobic effect.

Information, Table 2). The amount of variation in Δx_u for all tested conditions is small in comparison with the size difference between the DMSO and water molecules, thus indicating that no DMSO molecules bridge the gap between the force-bearing beta strands of GB1 in the unfolding transition state.

We thus fixed Δx_u to its mean value and performed a single-parameter fit of k_u^0 across all examined DMSO concentrations. The resulting spontaneous dissociation rate values showed a clear inverse correlation with DMSO concentration (Figure 2b). Accordingly, a consistent decreasing trend of the computed force-dependent unfolding rate was observed in force clamp experiments (Supporting Information Section 4, Figure S1). These observations demonstrate that the height of the unfolding transition barrier increases with increasing DMSO concentration. After correcting the data for the viscosity of DMSO–water mixtures[27] (Supporting Information, Section 3), the general increasing trend of $\Delta\Delta G_u$ is still maintained, with some uncertainty only in the low-concentration range (Figure 4). Therefore, the increase in viscosity alone is not sufficient to explain the protective effect of DMSO on the native state of GB1 against unfolding.

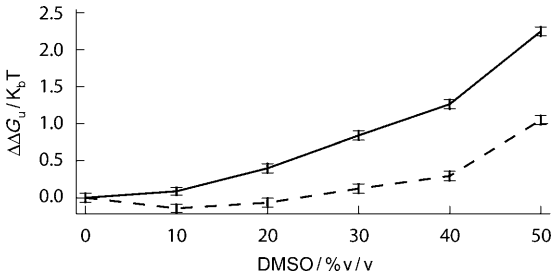

Figure 4. Height of the unfolding free energy barrier (by DMSO concentration) relative to the height in absence of DMSO (——), together with the viscosity-adjusted estimates (-----). Vertical error bars denote one standard deviation in both directions.

Similarly, we mapped the folding-energy landscape of GB1 on the mechanical reaction coordinate in the absence of DMSO and at a DMSO concentration of 30% v/v, extracting the distance between the unfolded state and the transition state Δx_f (see Figure 3) and the spontaneous folding rate k_f^0 (Supporting Information, Section 3). The data both in the absence and presence of DMSO is explained by the common value $\Delta x_f = 2.57$ (which is compatible with the previously published Δx_f value of GB1 in absence of DMSO[20]), resulting in $k_f^0 = 546 \pm 36$ in absence of DMSO and $k_f^0 = 693 \pm 64$ with 30% v/v DMSO. The osmolyte increased the height of the folding activation barrier by $\Delta\Delta G_f = (0.24 \pm 0.11)\, K_b T$, which becomes $\Delta\Delta G_f = (0.95 \pm 0.11)\, K_b T$ when correcting for viscosity effects (Supporting Information, Section 5.2)

Taken together, the results outlined above demonstrate that the protecting osmolyte DMSO is able to slow down the unfolding kinetics of the globular domain GB1 by raising the free energy of the transition state with respect to that of the native state. In particular, they demonstrate that the above effect is obtained by an indirect mechanism without any apparent complexation of the osmolyte to the protein in the transition state. Moreover, DMSO also accelerates the folding rate of GB1 by raising the free energy of the unfolded state with respect to the transition state. At the same time, the fact that Δx_u and Δx_f values remained constant across all tested experimental conditions implies that DMSO does not change the force dependence of the folding and unfolding kinetics, showing that its mode of action is independent of the effects of mechanical tension on the energy landscape of the protein.

According to the osmophobic model, a protecting osmolyte disfavors backbone solvent hydrogen bonding, and therefore it predominantly destabilizes structural states with a higher number of such bonds, for example, the unfolded state. If this is true, it would be expected that 1) protecting osmolytes should act on all the conformations assumed by the protein, and 2) the magnitude of their effect should be proportional to the amount of backbone solvent hydrogen bonds therein.[28] Obviously, the number of intrapeptide bonds is higher in the native state of a globular protein than in its unfolded state, while its transition state generally contains an intermediate number of such bonds.[29] Accordingly, a protecting osmolyte should mostly destabilize the unfolded state, but also to a lesser extent the transition state.[30] All of the above considerations collectively suggest that if an indirect, backbone-based osmophobic effect is acting, then the thermodynamic destabilization effect of the unfolded state should come to be through a kinetic protection against unfolding (that is, a slower spontaneous unfolding rate), together with a kinetic facilitation of folding (that is, a faster spontaneous folding rate; Figure 3). The results of the SMFS experiments described above are in perfect accord with this picture.

The ability of another protecting osmolyte, glycerol, to slow down the unfolding kinetics of ubiquitin[13] and the I27 immunoglobulin module of human cardiac titin[14] was previously demonstrated by single-molecule mechanical studies. In both cases, the osmolyte molecules were found to actively participate in the transition-state structure. However, similarly to what we observed with GB1 in DMSO–water mixtures, a kinetic protection against unfolding was observed in the two mentioned experiments[13,14] across a wide range of glycerol concentrations. As the protection resulted partly from the indirect, backbone-based osmophobic effect and partly from the more specific β-strand bridging phenomenon, from these previous experiments it was not possible to numerically evaluate or even claim evidence for the osmophobic effect. Preliminary experiments performed by us show that the solvent-bridging phenomenon observed with I27 in presence of glycerol does not occur with GB1, thus reinforcing the hypothesis that the formation of direct protein–osmolyte interactions at the transition state is not an universal phenomenon.

As deviations from the osmophobic mechanism are caused by direct complexation of the osmolyte with specific residues at the transition state (thus making alternative folding/unfolding pathways available), the fold complexity of a given protein may influence its susceptibility to show such deviations. Therefore, we expect that simple and small

proteins with low-fold complexity, which lack any folding/unfolding intermediates (as the GB1 domain examined in this study), should thus be less prone to show deviations from a purely osmophobic behavior under the effect of protecting osmolytes.

In summary, using a combination of SMFS-based experimental strategies we presented to the best of our knowledge the first single-molecule evidence of a protecting osmolyte slowing down the unfolding kinetics of a globular protein while concurrently accelerating its folding rate, independently of the mechanical tension applied and without any complexation in the unfolding transition state. This observation serves as evidence for a purely indirect backbone-based mechanism for the osmophobic effect.[1,4,7]

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