Retraction

CHEMISTRY

The authors note the following: “We wish to retract our article because interpretation of the experimental data was based on incorrect calibration of the apparatus in viscous solutions and there is no basis for the major conclusions of the study on the structure of the transition state for mechanical unfolding.”

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Probing osmolyte participation in the unfolding transition state of a protein

Lorna Dougan, Georgi Z. Genchev, Hui Lu, and Julio M. Fernandez

Understanding the molecular mechanisms of osmolyte protection in protein stability has proved to be challenging. In particular, little is known about the role of osmolytes in the structure of the unfolding transition state of a protein, the main determinant of its dynamics. We have developed an experimental protocol to directly probe the transition state of a protein in a range of osmolyte environments. We use an atomic force microscope in force-clamp mode to apply mechanical forces to the protein and obtain force-dependent rate constants of protein unfolding. We measure the change in the unfolding transition state, $\Delta \kappa_{u}$, along a 1D reaction coordinate imposed by mechanical force. We find that for the small osmolytes, ethylene glycol, propylene glycol, and glycerol, $\Delta \kappa_{u}$ scales with the size of the molecule, whereas for larger osmolytes, sorbitol and sucrose, $\Delta \kappa_{u}$ remains the same as that measured in water. These results are in agreement with steered molecular dynamics simulations that show that small osmolytes act as solvent bridges in the unfolding transition state structure, whereas only water molecules act as solvent bridges in large osmolyte environments. These results demonstrate that novel force protocols combined with solvent substitution can directly probe angstrom changes in unfolding transition state structure. This approach creates new opportunities to gain molecular level understanding of the action of osmolytes in biomolecular processes.

Solvent composition is actively modulated in vivo providing a diverse and optimized environment for biological processes (1). Solvent molecules facilitate necessary structural and dynamic arrangements, permit rapid conformational changes, catalyze chemical reactions, and mediate the self-assembly of biological molecules (2–4). There has been much effort to understand the role of the solvent environment on the behavior of proteins (5–9). In particular, studies have focused on understanding the function of osmolytes, small organic compounds that affect protein stability and are ubiquitous in living systems (5–8, 10, 11). Despite much effort, studies continue to attempt to find a universal molecular theory that can explain the mechanism by which osmolytes interact with proteins to affect protein stability (11). Although experiments have revealed a wealth of information regarding the thermodynamics of protein folding, very little is known about the role that solvent molecules play on the structure of the folding/unfolding transition state of a protein, which is the main determinant of protein dynamics (12).

Single-molecule force spectroscopy has emerged as a powerful tool to probe transition states in a protein (13). This technique is used to apply a mechanical force to a single protein, causing the protein to unfold and extend along a well-defined reaction coordinate: the end-to-end length of the protein (14). Along this unfolding pathway, a mechanically resistant transition state determines the force-dependent rate of unfolding, $\kappa_{u}(F)$, easily measured with force spectroscopy techniques. The force dependency of the unfolding rate is typically fit with an Arrhenius term that measures properties of the unfolding transition state. In its simplest representation, the unfolding transition state is characterized by two parameters: the size of its activation energy barrier, $\Delta G_{u}$, and the elongation of the protein necessary to reach the transition state, $\Delta \phi_{u}$. Of particular interest are the force spectroscopy measurements of $\Delta \phi_{u}$ that provide a direct measure of the lengths of a transition state, which were hitherto unknown.

We have previously demonstrated that force spectroscopy can be used to measure the distance to the unfolding transition state of a protein $\Delta \phi_{u}$ (13, 15). Completing single-molecule protein unfolding experiments on a number of different proteins in water, we measured values of $\Delta \phi_{u}$ in the range of 1.7–2.5 Å (13, 15, 16). These values of $\Delta \phi_{u}$ are comparable to the size of a water molecule (17), suggesting that water molecules are integral components of the unfolding transition state. Steered molecular dynamics (SMD) simulations have complemented our observations by providing a detailed atomic picture of stretching and unfolding of individual protein domains (18). The simulations are carried out by fixing one terminus of the protein and applying external forces to the other terminus. Earlier SMD simulations of forced unfolding of the 27th immunoglobulin-like domain of cardiac titin (I27) protein suggested that when a stretching force is applied between the protein’s termini, resistance to unfolding originated from a set of hydrogen bonds between two parallel β-strands (A′ and G) of the protein structure (19, 20). These β-strands provide a “mechanical clamp” that must be broken before unfolding can occur. Because the hydrogen bonds in the mechanical clamp region are perpendicular to the axis of extension, they must rupture simultaneously to allow relative movement of the two termini. Earlier SMD simulations showed that the breakage of interstrand hydrogen bonds could be followed by bonding to water molecules that then formed bridges between the two separating strands (19, 20). Given that force spectroscopy experiments measure a $\Delta \phi_{u}$ comparable to the size of a water molecule, one way to interpret the experimental results is that the transition state structure is formed by water molecules bridging the gap between separating β-strands and taking the place of some of the broken interstrand hydrogen bonds (19). Indeed, recent SMD simulations on the protein ubiquitin have demonstrated that this protein also contains a mechanical clamp region, and water molecules play an integral role in the protein’s unfolding transition state structure. Furthermore, these studies showed that hydrophobic interactions in the surface residues of the mechanical clamp region regulated the insertion of water molecules prior to hydrogen bond breakage and subsequent unfolding of the protein (21). These earlier studies point to the importance of water in the force-induced unfolding of a protein.
and suggests a “solvent bridging” mechanism in the unfolding transition state structure of the protein.

We have recently used force-clamp spectroscopy to test this solvent bridging mechanism by measuring $\Delta u_x$ of the I27 protein in the presence of the protecting osmolyte, glycerol, and deuterium oxide (13, 15). Glycerol is a good hydrogen bonding molecule that is ubiquitous in living systems, known to enhance protein stability, and is larger in molecular size than water (22). Deuterium oxide forms stronger hydrogen bonds than water while having a similar size (23). Our experiments showed that upon replacement of water by the similarly sized deuterium oxide did not change the value of $\Delta u_x$ (15). These experiments, combined with SMD simulations, directly demonstrated that solvent molecules form part of the structure of the mechanical transition state of the I27 protein, acting as “solvent bridges.”

To establish whether solvent bridging is a unique feature of the osmolyte glycerol and to further understand the unfolding transition state structure of the I27 protein, we have greatly expanded our studies. We have completed an extensive series of single-molecule experiments of protein unfolding on the I27 protein in a range of osmolyte solutions: ethylene glycol, propylene glycol, sorbitol, and sucrose. These osmolytes (Fig. 1) are capable of forming hydrogen bonds and vary in size from 4.8 Å (ethylene glycol) to 8.8 Å (sucrose), making them excellent candidates to test our solvent bridging hypothesis. These osmolytes also provide a toolbox of molecules of varying size to probe the unfolding transition state structure of the I27 protein.

![Fig. 1.](image)

Fig. 1. A molecular toolbox for determining the role of osmolyte molecules in the unfolding transition state of a protein. Using an array of osmolytes of varying size and hydrogen bonding abilities, we test the importance of solvent bridging in the I27 protein. Force-clamp protein unfolding traces for the I27 protein at a constant force of 180 pN in (A) water and a range of osmolyte solutions at a concentration of 1M: (B) ethylene glycol, (C) propylene glycol, (D) glycerol, (E) sorbitol, and (F) sucrose. Each unfolding trace shows the characteristic staircase of unfolding events, with each step of 24 nm corresponding to the unfolding of one I27 module of the polyprotein.

**Results**

Using the experimental protocol of our previous studies (24), we constructed and expressed a polyprotein that consisted of eight identical repeats of the I27 protein, $I27_8$, the mechanical properties of which have been extensively characterized experimentally (24, 25) and also in silico using molecular dynamics techniques (18, 19). The use of polyproteins is advantageous in that they provide a clear mechanical fingerprint of our system of interest to distinguish them against a background of spurious interactions. They also provide us with a larger number of events per recording than otherwise possible with monomers (26). We measure the properties of the mechanical unfolding transition state of the I27 protein by measuring the force dependency of the unfolding rate of single $I27_8$ polyproteins. When a protein is subjected to an external force, its unfolding rate, $k_u$, is well described by an Arrhenius term of the form $k_u(F) = k_0 \exp(\Delta G_u / k_B T)$, where $k_0$ is the unfolding rate in the absence of external forces, $F$ is the applied force, and $\Delta G_u$ is the distance from the native state to the transition state along the pulling direction (27). By measuring how the unfolding rate changes with an applied force, we can readily obtain estimates for the values of both $k_0$ and $\Delta G_u$ (13, 27). Given that $k_u = A \exp(\Delta G_u / k_B T)$, we can estimate the size of the activation energy barrier of unfolding $\Delta G_u$. From transition state theory, we assume a value of $10^9$ s$^{-1}$ for the prefactor, as has previously been used for mechanical unfolding of the I27 protein (24). The distance to transition state, $\Delta x_u$, determines the sensitivity of the unfolding rate to the pulling force and measures the elongation of the protein at the transition state of unfolding. Given that $k_u$ and $\Delta x_u$ reflect properties of the unfolding transition state, we expected that these variables would be strongly dependent on the solvent environment (13).

Under force-clamp conditions, stretching a polyprotein results in a well-defined series of step increases in length, marking the unfolding and extension of the individual modules in the chain (13). The size of the observed steps corresponds to the number of amino acids released by each unfolding event (28). Stretching a single $I27_8$ polyprotein in aqueous solution at a constant force of 180 pN results in a series of step increases in length of 24 nm (Fig. 1A). The time course of these events is a direct measure of the unfolding rate at 180 pN. To probe the role of the solvent environment in setting the structure of the unfolding transition state, we studied the effect of solvent substitution on the force dependency of the unfolding rate. We completed force unfolding experiments on the I27 protein in a range of osmolyte solutions: ethylene glycol, propylene glycol, sorbitol, and sucrose (Fig. 1B–F). These simple molecules are all capable of forming hydrogen bonds, and their thermodynamic properties have been extensively characterized (10, 11, 29, 30). Importantly, these osmolytes vary in size, making them excellent candidates to test our solvent bridging hypothesis and to probe the unfolding transition state structure of the I27 protein (11).

For each osmolyte solution we measure the unfolding rate of the I27 protein by fitting a single exponential to an average of 30 traces similar to the ones shown in Fig. 1 (27). Previous work on the protein ubiquitin has shown that a single exponential fit captures 81% of the unfolding events of the protein and thus represents a reasonable measure of the unfolding rate (31, 32). The summation and normalization of unfolding traces are shown in Fig. 2A–D for I27 protein unfolding in a solution of ethylene glycol, propylene glycol, sorbitol, and sucrose at a range of forces from 140–200 pN. We define the unfolding rate as $k_u(F) = 1/\tau(F)$, where $\tau(F)$ is the time constant of the exponential fit to the averaged unfolding traces, shown in Fig. 2A–D (27). Furthermore, we obtain an estimate of the standard error of $k_u(F)$, using the bootstrapping technique (33, 34) (see SI Text). We repeated these measurements and obtained the force dependency of the
unfolding for each osmolyte solution: ethylene glycol, propylene glycol, sorbitol, and sucrose at a range of concentrations.

In Fig. 3 we show the natural logarithm of $k_u$ as a function of applied force for each osmolyte for a range of concentrations up to the solubility limit of the osmolyte. We fit the Arrhenius rate equation to the data (Fig. 3) to obtain $k_u^0$ and $\Delta x_u$. It is apparent from Fig. 3 A–D that the introduction of increasing amounts of osmolyte decreases the value of $k_u^0$ compared with that of water (black data points and line in Fig. 3). In addition to the observed changes in $k_u^0$, there are striking differences in the slope ($\Delta x_u/k_B T$) of the force dependency of unfolding in Fig. 3. In the case of ethylene glycol (Fig. 3A) and propylene glycol (Fig. 3B) the slope increased, resulting in an increase in the distance to transition state from $\Delta x_u = 2.5 \text{ Å}$ in aqueous solution and up to $3.65 \pm 0.1 \text{ Å}$ in 62% by weight ethylene glycol and $4.1 \pm 0.1 \text{ Å}$ in 60% by weight propylene glycol, respectively. Interestingly, for the larger osmolytes, sorbitol and sucrose, the slope of the force dependency of the unfolding rate showed no change compared with that measured in water (Fig. 3 C and D).

In Fig. 4 we show the full picture of the effect of osmolytes on $\Delta G_u$ (Fig. 4A) and $\Delta x_u$ (Fig. 4B), where $\Delta G_u$ is calculated using $k_u^0 = A \exp(\Delta G_u/k_B T)$. For all the osmolytes studied, $\Delta G_u$ is found to increase with increasing weight fraction of osmolyte compared with that measured in water (Fig. 4A). Thus, the osmolytes used in this study stabilize the protein I27 against the denaturation induced by mechanical force. In the case of the small osmolytes, ethylene glycol and propylene glycol, $\Delta G_u$ increases significantly from $23.11 \text{ kcal mol}^{-1}$ in aqueous solution to $27.08 \text{ kcal mol}^{-1}$ in aqueous ethylene glycol and $28.80 \text{ kcal mol}^{-1}$ in aqueous propylene glycol. For the larger osmolytes $\Delta G_u$ is also observed to increase, but to a lesser extent with a $\Delta G_u$ of $24.67 \text{ kcal mol}^{-1}$ in aqueous sorbitol and $24.21 \text{ kcal mol}^{-1}$ in aqueous sucrose. Thus, small osmolytes seem to be more effective in stabilizing the I27 protein against mechanical denaturation. In Fig. 4B we show the measured value of $\Delta x_u$ for each osmolyte solution for a range of different concentrations, in weight fraction of osmolyte. Strikingly, for the small osmolytes, ethylene glycol and propylene glycol, we observe that the value of $\Delta x_u$ increases in a sharply nonlinear manner. For ethylene glycol (squares), $\Delta x_u$ increases rapidly and then saturates at a value of $3.65 \text{ Å}$, whereas for propylene glycol $\Delta x_u$ increases rapidly and saturates at a value of $4.0 \text{ Å}$. This is in good agreement with our earlier experimental observations with aqueous glycerol where we measured a sharply nonlinear increase in $\Delta x_u$ (13). Contrary to the behavior of the small osmolytes, our experiments of aqueous solutions of sorbitol and sucrose show a very different trend in the measured value of $\Delta x_u$. As before, we measured the value of $\Delta x_u$ in sorbitol and sucrose solutions by fitting an Arrhenius term to the force dependency of the unfolding rate in Fig. 3 C and D. Our measurements showed that $\Delta x_u$ remained unchanged across the entire concentration range measured (Fig. 4B). Indeed, the measured value of $\Delta x_u$ is $2.5 \text{ Å}$ for both sorbitol (triangle) and sucrose (circles) at all

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**Fig. 2.** The average time course of unfolding was obtained by summation and normalization of unfolding traces. Multiple trace averages ($n > 30$ in each trace) of unfolding events measured for I27 in (A) 44% by weight ethylene glycol solution at constant forces of 220, 200, 180, and 160 pN; (B) 30% by weight propylene glycol solution at constant forces of 200, 180, 160, and 140 pN; (C) 35% by weight sorbitol solution at constant forces of 200, 180, 160, and 140 pN; (D) 34% by weight sucrose solution at constant forces of 220, 200, 180, and 160 pN.

**Fig. 3.** Semilogarithmic plot of the rate of unfolding of I27 as a function of pulling force in (A) 6% (squares), 32% (upward triangles), 44% (downward triangles), and 66% (diamonds) by weight ethylene glycol solution; (B) 10% (squares), 20% (upward triangles), 36% (downward triangles), and 60% (diamonds) by weight propylene glycol solution; (C) 15% (squares), 35% (upward triangles), 52% (downward triangles) by weight sorbitol solution; (D) 14% (squares), 34% (upward triangles), and 54% (downward triangles) by weight sucrose solution. For comparison, the force dependency of unfolding I27 in aqueous solution is shown on each graph (circle).
concentrations. This is the same value of $\Delta x_u$ that is measured in a water solution (13).

In Fig. 5A (squares) the maximum $\Delta x_U$ measured for each osmolyte is shown along with the molecular size of each osmolyte. For the smaller osmolytes, ethylene glycol, propylene glycol and from our earlier studies, glycerol (13), $\Delta x_U$ increases from that measured in water. Furthermore there is a correlation between the experimentally measured value of $\Delta x_U$ and osmolyte molecular size. For the larger osmolytes, sorbitol and sucrose, no such correlation between $\Delta x_U$ and molecular size is observed. We complemented our experimental measurements by exploring the mechanisms of solvent molecule participation in the unfolding transition state using SMD simulations. These SMD simulations provide a detailed atomic picture of stretching and unfolding of individual I27 protein domains (18, 19). Our simulations of forced unfolding of the I27 protein in ethylene glycol and sorbitol solutions showed that the resistance to unfolding originates from the hydrogen bonds between the $\beta$-strands (Fig. 5B). During unfolding, the solvent molecules attempt to break and bridge the backbone hydrogen bonds between the $\beta$-strands. In the simulations of ethylene glycol solutions, the larger size of the cosolvent and the solvent bridge it forms leads to a greater gap separating the $\beta$-strands (Fig. 5C). The simulations showed that the separation between the two strands is increased due to the insertion of the ethylene glycol solvent bridge. Our earlier SMD simulations of forced unfolding of the I27 protein in glycerol solutions showed that the larger size of this cosolvent and its ability to act as a solvent bridge could lead to a greater distance between the neighboring $A-G$ $\beta$-strands (13). In the case of sorbitol solutions, there was no evidence in the SMD simulations of a sorbitol molecule acting in the same way as ethylene glycol. There were cases when the end hydroxyl group could hydrogen bond with the $A'$ and $G$ $\beta$-strands (Fig. 5D). However, we found no evidence for a full sorbitol molecule solvent bridging between the $A'$ and $G$ $\beta$-strands of the I27 protein. Instead, the simulations predominately identified water molecules bridging the $A'$ and $G$ $\beta$-strands. Thus for the sorbitol SMD simulations we did not observe an increased separation between the $\beta$-strands.

Given that there is a multitude of possible transition state structures formed by water, osmolyte, and the protein backbone, there is no straightforward way to link a wider gap between the $\beta$-strands $A'$ and $G$ in the simulations and the experimentally measured values of $\Delta x_U$. In Fig. 5C and D, we define the pulling coordinate for the separating $\beta$-strands as the distance between the first amino acid of strand $A'$ (shown as a large blue sphere) and the last amino acid of strand $G$ (also shown as a large blue sphere). This distance gets longer as the two $\beta$-strands separate under a constant force, filling the gap with solvent molecules until a transition state is reached. In the SMD simulations the elongation of this separation of the $\beta$-strands up to the transition state is defined as the distance to the transition state $\Delta x_{A'-G}$. The crossing of the transition state is marked by an abrupt increase in the

![Fig. 5](image)

**Fig. 5.** The maximum $\Delta x_u$ measured using the experimental data for propylene glycol, ethylene glycol, sorbitol, and sucrose (squares) and $\Delta x_{A'-G}$ measured from the SMD simulations for ethylene glycol and sorbitol (circles). For comparison $\Delta x_u$ and $\Delta x_{A'-G}$ are also shown for glycerol and water (13). For the smaller osmolytes, ethylene glycol, propylene glycol, and glycerol, there is a clear correlation between measured $\Delta x_u$, $\Delta x_{A'-G}$ and molecular size. For the larger osmolytes, sorbitol and sucrose, no such correlation between $\Delta x_u$ or $\Delta x_{A'-G}$ and molecular size is observed and the value is $\Delta x_u$ is the same as that of water. (A) The 27th immunoglobulin-like domain of cardiac titin I27 protein (1TIT.pdb). Resistance to mechanical unfolding originates from six hydrogen bonds between two parallel $\beta$-strands $A'$ and $G$ (19). (C) Snapshot from an SMD simulation of forced unfolding of the I27 protein in ethylene glycol solution. Osmolyte bridging between the two parallel $\beta$-strands $A'$ and $G$ is observed leading to an increased separation of the two $\beta$-strands. (D) Snapshot from an SMD simulation of forced unfolding of the I27 protein in sorbitol solution. The sorbitol molecule does not insert between the two parallel $\beta$-strands but instead forms a hydrogen bond from the terminal hydroxyl. No increased separation of the two $\beta$-strands is observed.
Discussion
Recent studies have shown that the mechanical stability of a protein can be modulated by the presence of cosolvents in the surrounding environment. Mechanical unfolding experiments of the protein I27 in aqueous glycerol solutions measured a considerable increase in the mechanical stability of the protein, with the average unfolding force increasing by approximately 50% (13).

Similarly, in the presence of 30% dextran, a polysaccharide molecule, the average unfolding force of the protein ubiquitin increases by approximately 11% (35). Conversely, denaturing osmolytes such as guanidinium chloride have been shown to decrease the mechanical stability of the small protein GB1 (the B1 immunoglobulin-binding domain of protein G from Streptococcus) by approximately 80% in 2.25 M GdmCl compared with an aqueous solution (36).

Denaturing and protecting osmolytes therefore offer an attractive route to modulate the mechanical properties of a protein. Indeed, recent single-molecule atomic force microscopy (AFM) experiments have shown that naturally occurring protecting and denaturing osmolytes have profound effects on the mechanical folding pathways of polycystic kidney disease (PKD) domains (37).

This study demonstrated that protecting osmolytes such as sorbitol and trimethylamine N oxide are efficient in counteracting the effect of the denaturing osmolyte urea on the mechanical stability of PKD domains.

In the present study we have tested the origin of enhanced mechanical stability in a protein by developing an experimental approach that provides insight into the role of osmolytes in the unfolding transition state structure of a protein, the main determinant of protein kinetics. The unfolding transition state of a protein under force can be defined as that structure that, taken as a starting point, leads to either full unraveling or to a stable protein under force can be defined as that structure that, taken as a starting point, leads to either full unraveling or to a stable structure, regardless of its lifetime. Thus, our finding that the near doubling of Δτ is a highly significant because it directly points to an integral structural role of these osmolytes in the unfolding transition state of the I27 protein.

Methods
Protein Engineering and Purification. We constructed an eight-domain N-C-linked polypeptide of I27, the 27th Ig-like domain of cardiac titin, through successive cloning in modified pT7Blue vectors and then expressed the gene using vector pQE30 in Escherichia coli strain BL21(DE3) (24). The polypeptide construct was finally purified by histidine metal-affinity chromatography with Talon resin (Clontech) and by gel filtration using Superdex 200 HR column (GE BioSciences).

Force Spectroscopy. Force-clamp AFM experiments were completed at room temperature using a homemade setup under force-clamp conditions described elsewhere (14). Experiments were carried out in a sodium phosphate buffer solution (specifically, 50 mM sodium phosphate (Na2HPO4 and NaH2PO4), and 150 mM NaCl, pH = 7.4) with the desired weight percentage of the cosolvent. Samples of propylene glycol (99%), ethylene glycol (99%), glycerol (99%), sorbitol (99%), and sucrose (99%) were obtained from Sigma-Aldrich and used without additional purification.

Steered Molecular Dynamics Simulations. We completed SMD simulations of I27 unfolding in ethylene glycol and sorbitol using a method described...
previously (13). Each simulation started from the equilibrated protein structure with the corresponding solvent. Constant forces are added to the C termini of the I27 protein, described in Table S1 and in SI Text. The coordinate PDB file of titin’s I27 protein (1TIT.pdb) was used. A molecular structure file was generated for the full system using visual molecular dynamics (VMD) (47), and the solvent environment was modeled explicitly.


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