

The Important Roles of Water in Protein Folding: an Approach by Single Molecule Force Spectroscopy

Bo Cheng^{a, b} and Shu-Xun Cui^{b*}

^a School of Mechanical Engineering, North University of China, Taiyuan 030051, China

^b Key Lab of Advanced Technologies of Materials, Ministry of Education of China, Southwest Jiaotong University, Chengdu 610031, China

Electronic Supplementary Information

Abstract The single-chain elasticity of a completely unfolded protein ((I27)₈, modules of human cardiac titin) is studied in different liquid environments by the atomic force microscopy (AFM)-based single molecule force spectroscopy (SMFS). The experimental results show that there is a clear deviation between the force curves obtained in the aqueous and nonaqueous environments. Such a deviation can be attributed to the additional energy consumed by the rearrangement of the bound water molecules around the chain of the completely unfolded (I27)₈ chain upon stretching in aqueous solution, which is very similar to the partial dehydration process from a denatured/unfolded to a native/folded protein. Through the analysis of the free energy changes involved in protein folding, we conclude that it is due to the weak disturbance of water molecules and the special backbone structures of proteins that the self-assembly of proteins can be achieved in physiological conditions. We speculate that water is likely to be an important criterion for the selection of self-assembling macromolecules in the prebiotic chemical evolution.

Keywords SMFS; Protein; Folding; Water

Citation: Cheng, B.; Cui, S. X. The Important Roles of Water in Protein Folding: an Approach by Single Molecule Force Spectroscopy. *Chinese J. Polym. Sci.* 2018, 36(3), 379–384.

INTRODUCTION

Water, one of the most important substances for lives on the Earth, is vital both as a solvent in which many of the organism's solutes dissolve and as an essential part of many metabolic processes within the organism^[1, 2]. Water is treated as the “twenty-first” amino acid because of its important role in biomolecular structures, dynamics and functions^[3]. The water-dependent hydrophobic interactions are one of the driving forces for protein folding and other biological processes (*e.g.*, aggregation of amphiphilic lipids into membranes)^[4, 5]. For proteins, the aqueous solution provides an appropriate environment, in which they adopt their native structures and demonstrate the relevant functions^[2].

It is expected that water molecules play a vital role in the formation of proper structures of proteins. So far, most of the related investigations on proteins were mainly carried out in aqueous surroundings. However, it is difficult to discern effects of water molecules alone in aqueous media^[6]. An alternative choice is to carry out the related studies in nonaqueous solvents. It is well-known that proteins are soluble in some polar organic solvents (*e.g.*, keep the folded structures in glycerol), but denatured in some polar organic

solvents (*e.g.*, adopt partially folded structures in ethylene glycol, and relatively unfolded structures in DMF and DMSO)^[7]. Besides these, the solubility of a protein in nonpolar organic solvents is almost zero^[8], which indicates that there are no specific interactions between the protein and the nonpolar organic solvents. Therefore, if proteins can be studied in a neat nonpolar organic solvent, it will be helpful to investigate the actual effects of water molecules on the structures of proteins. However, due to the insolubility, it is difficult to study the behaviors of proteins in nonpolar organic solvents by the traditional methods.

Recently, atomic force microscopy (AFM)-based single molecule force spectroscopy (SMFS) has been developed into a powerful tool to investigate the single-molecule mechanical properties of proteins under an external force^[9–11]. One of the key features of AFM is that the target molecules can be operated in different environments, no matter whether they are soluble or not. It has been reported that, when one protein is pulled in a nonpolar organic solvent, its native structure can be unfolded into a polypeptide chain without special structure^[12], which demonstrates that the water molecules play a crucial role to protect the native structure from unfolding. However, the thermodynamics details of the effect of water molecules on the structures of proteins are still unclear. Therefore, it is necessary to study the single-chain elasticity of a completely unfolded protein (*i.e.*, in a “polypeptide” state) in nonpolar solvents and aqueous

* Corresponding author: E-mail cuishuxun@swjtu.edu.cn

Invited paper for special issue of “Supramolecular Self-Assembly”

Received September 23, 2017; Accepted November 2, 2017; Published online December 7, 2017

solutions.

Here in this study, we select the (I27)₈ Ig modules of human cardiac titin as the model system of protein, which have been studied in detail by using NMR^[13], steered molecular dynamics^[14], and SMFS^[15–17]. We employ SMFS to detect the single-chain mechanics of one completely unfolded (I27)₈ in nonpolar solvents and aqueous environment, respectively. We find that, there is a clear energy difference between the force-extension curves (in brief, force curves) obtained in the two conditions, which can be calculated to be $1.25 k_B T/\text{unit}$.

EXPERIMENTAL

Materials and Sample Preparation

The (I27)₈ specimen was purchased from Athena Environmental Sciences, Inc. (Baltimore, MD). It was a 94 kDa synthetic polyprotein composed of eight repeats of the I27 domain of human titin protein. Deionized (DI) water ($> 15 \text{ M}\Omega\cdot\text{cm}$) was used when water was involved. All other chemical reagents were purchased from Sigma and analytically pure, if not mentioned otherwise. For the sample preparation, (I27)₈ was diluted with PBS (pH = 7.4) to a final concentration of $100 \mu\text{g/mL}$. A few drops of the (I27)₈ solution were deposited onto a freshly evaporated gold surface for 12 h, followed by rinsing with PBS to remove the loosely adsorbed molecules^[18]. After dried by air flow, the sample was ready to be used. The poly-L-lysine (MW $\geq 3 \times 10^5$, Sigma Corp.) was also diluted with PBS buffer to the final concentration of $100 \mu\text{g/mL}$. The preparation of poly-L-lysine sample for the force measurements was similar to that of (I27)₈.

Force Measurements

All force experiments were performed on a commercial AFM (MFP-3D, Oxford Instruments). Prior to the measurements, a drop of liquid was injected between the V-shaped Si₃N₄ AFM cantilever (Bruker Corp., CA) and the sample. Then during the AFM manipulation, the data were recorded at the same time and converted to force curves subsequently. The spring constant of each AFM cantilever was calibrated by the thermo-excitation method, ranging from 20 pN/nm to 50 pN/nm^[19]. The stretching velocity applied in this study was $2.0 \mu\text{m/s}$, if not mentioned otherwise. The experimental details of the AFM instrumentation can be found elsewhere^[9, 20, 21].

RESULTS AND DISCUSSION

The Single-chain Mechanics of Unfolded (I27)₈ in Nonpolar Solvents

At the first step, we measured the single-chain mechanics of (I27)₈ in a nonpolar organic solvent—octylbenzene (Fig. S1, in electronic supplementary information, ESI), which is a poor solvent for proteins. As described in the literature^[12], the characteristic “sawtooth” profiles, which correspond to the successive unfolding events of the protein domains, are not observed at all. Only “single peak” can be detected in each force curve (see Fig. 1a). In a typical force curve, the force rises monotonically with extension until the molecular bridge

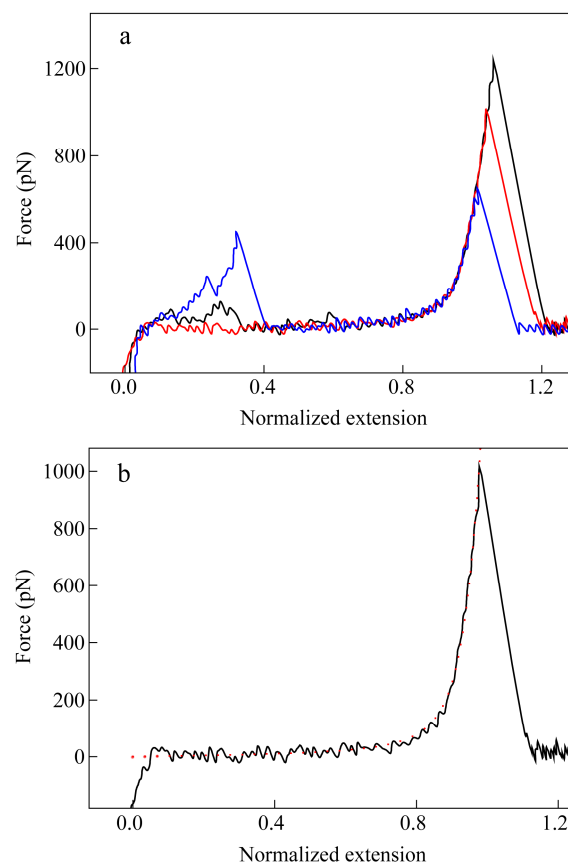


Fig. 1 (a) Normalized force curves obtained from an (I27)₈ sample in octylbenzene; (b) The comparison of force curve of (I27)₈ obtained in octylbenzene (black line) and the QM-WLC model fitting curve with $l_p = 0.38 \text{ nm}$ (red dotted line)

between the tip and substrate is broken. After normalization, these force curves can be superposed very well with each other, indicating that the force curves represent the single-chain mechanics of (I27)₈ in octylbenzene^[22, 23]. One can find that these force curves are similar to the single-chain mechanics of a random-coiled polymer without special structure. Thus, we can conclude that the (I27)₈ molecule has been completely unfolded into a polypeptide chain upon stretching in octylbenzene^[12]. After the sample preparation, there is a nano-layer of water adsorbed on the sample surface^[6]. When the tip moves away from the gold surface, one (I27)₈ molecule is pulled from aqueous environment to nonpolar organic solvent. It is well known that the hydrophobic interactions play an important role during the protein folding^[4, 5, 24, 25], which should be absent in octylbenzene because there is almost no water. When the (I27)₈ chain is pulled from water to octylbenzene, it is expected that the folded structure will be unstable. In such a case, the thermal fluctuation (at the level of $k_B T$) at room temperature could be strong enough to destroy the native supramolecular structure of a protein. Therefore, these force curves in Fig. 1(a) represent the single-chain mechanics of a completely unfolded (I27)₈ in octylbenzene.

To strengthen this conclusion, a recently developed theoretical model, QM-WLC model, is utilized to fit the experimental force curve. As shown in Fig. 1(b), the force

curve can be superposed very well with the fitting curve at $l_p = 0.38$ nm, which is identical to the length of one residue in a polypeptide chain. This result indicates that the force curve represents the pure elastic behavior of a completely unfolded (I27)₈ chain in octylbenzene.

In addition, a typical polypeptide, poly-L-lysine, is chosen as the sample for the force measurements. By measuring the single-chain mechanics of poly-L-lysine in octylbenzene, it can be found that the force curve can be superposed well with that of (I27)₈ obtained in octylbenzene, shown in Fig. S2 (in ESI). Because the poly-L-lysine chain adopts a random-coiled conformation, the (I27)₈ molecule should adopt a similar conformation in octylbenzene.

The Single-chain Mechanics of Unfolded (I27)₈ in Aqueous Solution

In one cycle of a typical SMFS force measurement, two force curves are obtained, *i.e.*, the stretching force curve and the relaxing one. For a common polymer without special structure, the two force curves should be superposed very well, both of which could reflect the single-chain elastic behaviors of the polymer chain. As for a protein with a fine supramolecular structure, the stretching force curve represents the consecutive unfolding events of a native protein, but the relaxing force curve of the completely unfolded protein reflects the single-chain elastic properties of an unfolded “polypeptide” chain^[26].

Then, we obtain the relaxing force curves of a completely unfolded (I27)₈ in PBS buffer (Fig. S3, in ESI), and find that these force curves are all monotonically increased with extension, which is similar to the single-chain mechanics of a common polymer without special structure. After normalization, it can be found that these relaxing force curves are superposed very well (see Fig. 2). This result indicates that the relaxing force curves can represent the single-chain mechanics of a completely unfolded (I27)₈ in PBS buffer.

In order to exclude the influence of loading rates, we also obtain the relaxing force curves of a completely unfolded (I27)₈ at different stretching velocities in PBS buffer. From Fig. S4 (in ESI), one can find that these force curves can be superposed very well with each other, which implies that the relaxing process is carried out in a quasi-equilibrium condition. In addition, the single-chain mechanics of

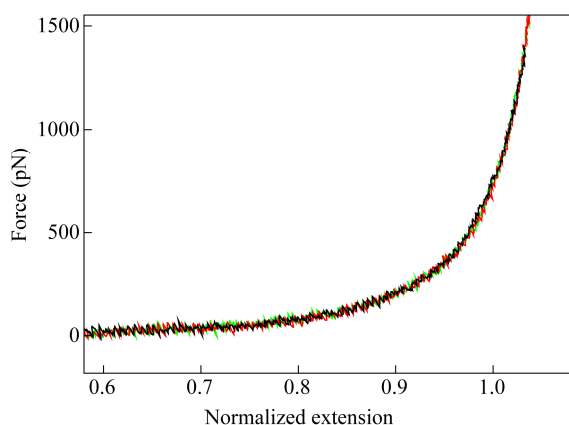


Fig. 2 The normalized relaxing force curves of a completely unfolded (I27)₈ obtained in PBS buffer

poly-L-lysine in PBS buffer was measured to further confirm the results. After normalization, the stretching force curves of poly-L-lysine are superposed very well with the relaxing force curves of (I27)₈ obtained in PBS buffer (see Fig. S5 in ESI). This result indicates that the single-chain mechanics of a completely unfolded (I27)₈ is identical to that of a random-coiled poly-L-lysine in aqueous media, which is reasonable because they share the same backbone^[27].

The Difference of Single-chain Mechanics of Unfolded (I27)₈ in Aqueous and Nonaqueous Environments

Subsequently, we compare the single-chain mechanics of a completely unfolded (I27)₈ obtained in nonpolar solvents and PBS buffer, respectively. From Fig. 3(a), it can be observed that the two force curves are superposed very well at low and high forces. However, there is a clear deviation between them in the middle force region. To find out the possible reason, two different kinds of aqueous media are used, including DI water and 1 mol/L NaCl solution, to repeat the force measurements. As shown in Fig. S6 (in ESI), there is no evident difference among the force curves obtained in these aqueous media, which indicates that the single-chain mechanics is salt-independent. Therefore, we suppose that the bound water around the chain may be a potential explanation^[23, 28].

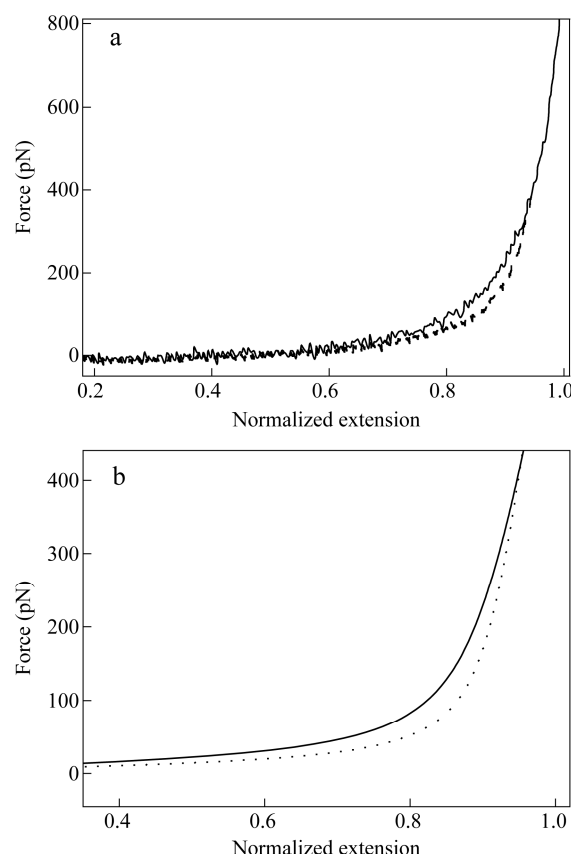
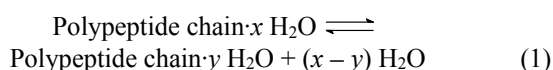


Fig. 3 The comparison of normalized force curves of a completely unfolded (I27)₈ obtained in aqueous environment (upper line) and nonpolar organic solvents (lower line): (a) The original force curves obtained from experiments; (b) The two force curves smoothed and enlarged to show a clear deviation between them

The above experimental results show that poly-L-lysine and a completely unfolded (I27)₈ share the same single-chain mechanics, as long as the force measurements are carried out under the same condition. Considering that the sequences of poly-L-lysine and (I27)₈ are totally different, we suppose that all polypeptides and completely unfolded proteins share the same single-chain mechanics under the same condition. Thus, a random-coiled polypeptide chain can be used as the model system of the completely unfolded protein. It is known that there are many hydrogen-bonding acceptors and donors in one polypeptide chain. Thus, some water molecules can bind to the polypeptide chain by forming hydrogen-bonds in aqueous solution. When the polypeptide chain is in a fully stretched state, due to the limitations in chain conformation, there will be fewer bound water molecules per repeating unit than that in the free coil state. That is, the polypeptide chain would lose bound water molecules gradually during the force stretching. This rearrangement of the bound water molecules will consume additional energy besides those contributions from the “pure” elasticity (*e.g.*, the single-chain elasticity in nonpolar organic solvents). In other words, the stretching process of a random-coiled polypeptide chain in aqueous solution will consume more energy than that consumed in nonpolar organic solvents, which is calculated to be $1.25 k_B T/\text{unit}$ ($3.1 \text{ kJ}/(\text{mol residue})$), as reflected in the deviation shown in Fig. 3^[22].

The Free Energy Change Involved in the Protein Folding in Aqueous Solution

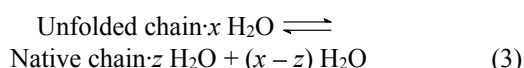
According to the above results, the water rearrangement around the completely unfolded (I27)₈ chain upon AFM stretching can be regarded as a partial dehydration process, see Eq. (1) below:



It is known that the folding-unfolding of proteins is a reversible process. This means that the native and unfolded states exist simultaneously in a dynamic equilibrium^[29], which can be described by Eq. (2):

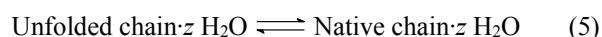
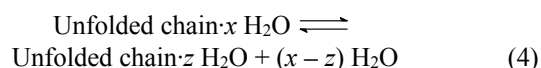


Generally speaking, a protein favors the native state in physiological conditions. If the physiological condition is changed, *e.g.*, in a 6 mol/L Guanidinium chloride solution, the equilibrium will move toward the unfolded state. It is known that both the native state and the unfolded state are hydrated in the aqueous solution, and the binding sites with water in the unfolded state is more than that in the native state^[30]. Thus, a partial dehydration process will occur while the native structure is formed. Then, Eq. (2) should be corrected as follows:



According to the literature, a typical value for the Gibbs free energy change for Eq. (3) (ΔG_3) is about $-0.5 \text{ kJ}/(\text{mol residue})$ ^[16, 31–34]. The formation of protein native structure can be separated into two steps, including the partial

dehydration and the supramolecular self-assembly, as described by the two equations below:



In the above two steps, all the water rearrangement is completed in Eq. (4), and all the self-assembly occurs in Eq. (5), which are a non-spontaneous ($\Delta G_4 > 0$) and a spontaneous process ($\Delta G_5 < 0$), respectively. It is clear that for the total process, which is a favorable reaction under physiological conditions, the Gibbs free energy change is negative, *i.e.*, $\Delta G_3 = \Delta G_4 + \Delta G_5 < 0$. Thus, the Gibbs free energy change in the process of partially removing the hydration shell of the unfolded chain must be compensated by that of the formation of the native structure, *i.e.*, $\Delta G_4 < -\Delta G_5$. However, ΔG_4 (or ΔG_5) is neither a ready datum in references, nor a value that can be measured easily.

Interestingly, it can be observed that the process of partial dehydration in Eq. (4) is very similar to the process of the loss of bound water upon AFM stretching in Eq. (1). Then, it is expected that the Gibbs free energy changes of these two equations are very close, *i.e.*, $\Delta G_1 \approx \Delta G_4 \approx 3.1 \text{ kJ}/(\text{mol residue})$. Thus, we can calculate that ΔG_5 is about $-3.6 \text{ kJ}/(\text{mol residue})$, which is not far from zero. In such a case, the value of ΔG_4 is vital to the process of Eq. (3). If ΔG_4 is a much larger value (*e.g.* $> 3.6 \text{ kJ}/(\text{mol residue})$), the whole process of protein folding will not be favorable, because ΔG_3 would be a positive value. Although the water rearrangement upon AFM stretching also occurs in other macromolecule/water systems, like the poly(*N*-vinyl-2-pyrrolidone)/water system ($13.0 \text{ kJ}/\text{mol unit}$)^[35] or the poly(ethylene-glycol)/water system ($7.2 \text{ kJ}/\text{mol unit}$)^[36], it is found that biomacromolecules (such as protein and DNA^[2]) cost lower energy among them. Therefore, the self-assembly of proteins in physiological conditions can be achieved due to the weak disturbance of water molecules around the unfolded chain.

The Backbone Structure of Proteins and the Prebiotic Chemical Evolution

As discussed above, water plays a key role in the self-assembly of proteins. On one hand, it is the weak disturbance of water molecules that ensures the stability of the folded structure in aqueous media. On the other hand, by shaving off the water molecules, proteins will be destabilized and tend to unfold. Another fact is that, there are many kinds of water soluble macromolecules, but only a few can form stable folded structures. This may imply that proteins are somewhat special in the backbone structure.

We suppose that the specific backbone structure of proteins is not a result of chance. It is more likely to be the final result of the natural selection. We conceive a possible route of the prebiotic chemical evolution as described below.

Because of the complexity, we attempt to fractionize the whole prebiotic chemical evolution into 6 levels. First, the primitive Earth is an inorganic world. There are gas mixtures, water, and inorganic molecules (Level 1). Then, the inorganic compounds may be converted into the organic monomers by

the Miller-Urey process and other possible routes^[2] (Level 2). Afterwards, many macromolecules can be generated by polymerization reactions (Level 3). The reactions involved from Level 1 to Level 3 are in a dynamic equilibrium because of the reversibility under certain conditions. To prevent the degradation into monomers, these macromolecules must form stable supramolecular structures (Level 4). However, water is a strong solvent, which usually has remarkable impact on the solute molecules. With the strong interference from water, only a few macromolecules can form stable supramolecular structures in aqueous solution. As a result of long-time natural selection, the backbone structure of proteins has survived (Level 5). Note that DNA and RNA also have stable supramolecular structures in aqueous media^[2]. With RNA, DNA and proteins, the “central dogma” may be realized in a primitive cell (Level 6).

During the whole chemical evolution, water can be regarded as the most important substance. As a reactant and the only solvent, it directly participates in the earlier prebiotic chemical evolution. Also, as a “selector”, most of the macromolecules in the Level 3 were screened out by the water environment. Therefore, water, as the primary environment of the earth, is not only a support of life, but also a restraint of the form of life. It is interesting that the prebiotic chemical evolution may also run in a Darwin fashion^[2, 37].

CONCLUSIONS

In summary, we have measured the single-chain elasticity of a completely unfolded protein ((I27)₈) in aqueous environment and nonpolar organic solvents respectively by using SMFS. By comparing these force curves, it is found that there is a clear deviation between them in the middle force region, which is calculated to be 3.1 kJ/(mol residue) in energy. The deviation is attributed to the rearrangement of water molecules around the completely unfolded (I27)₈ chain upon stretching in an aqueous solution, which does not exist in nonpolar organic solvents. Through the energy analysis of protein folding, we find that the partial dehydration process of a protein before folding is very similar to the process of the loss of bound water of a protein in aqueous solution upon AFM stretching. It is the weak disturbance of water that enables the folding of protein in physiological conditions. We suppose that, the backbone structures of proteins are the final results of natural selection in the early chemical evolution, where water acted as the selector. It is greatly anticipated that this notion will cast new light on the origin of life in the future.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at: <https://doi.org/10.1007/s10118-018-2082-2>.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (Nos. 21574106 and 21604074) and the Sichuan Province Youth Science and Technology Innovation Team (Nos. 2016TD0026 and 2017JQ0009).

REFERENCES

- 1 Ball, P. Water as an active constituent in cell biology. *Chem. Rev.* 2008, 108(1), 74–108.
- 2 Cui, S. The possible roles of water in the prebiotic chemical evolution of DNA. *Phys. Chem. Chem. Phys.* 2010, 12(35), 10147–10153.
- 3 Levy, Y.; Onuchic, J. N. Water mediation in protein folding and molecular recognition. *Annu. Rev. Biophys. Biomol. Struct.* 2006, 35, 389–415.
- 4 Papoian, G. A.; Ulander, J.; Eastwood, M. P.; Luthey-Schulten, Z.; Wolynes, P. G. Water in protein structure prediction. *Proc. Natl. Acad. Sci. U. S. A.* 2004, 101(10), 3352–3357.
- 5 Scatena, L. F.; Brown, M. G.; Richmond, G. L. Water at hydrophobic surfaces: weak hydrogen bonding and strong orientation effects. *Science* 2001, 292(5518), 908–912.
- 6 Cui, S.; Yu, J.; Kuehner, F.; Schulten, K.; Gaub, H. E. Double-stranded DNA dissociates into single strands when dragged into a poor solvent. *J. Am. Chem. Soc.* 2007, 129(47), 14710–14716.
- 7 Knubovets, T.; Osterhout, J. J.; Klibanov, A. M. Structure of lysozyme dissolved in neat organic solvents as assessed by NMR and CD spectroscopies. *Biotechnol. Bioeng.* 1999, 63(2), 242–248.
- 8 Houen, G.; Svaerke, C.; Barkholt, V. The solubilities of denatured proteins in different organic solvents. *Acta Chem. Scand.* 1999, 53(12), 1122–1126.
- 9 Hugel, T.; Seitz, M. The study of molecular interactions by AFM force spectroscopy. *Macromol. Rapid Commun.* 2001, 22(13), 989–1016.
- 10 Janshoff, A.; Neitzert, M.; Oberdorfer, Y.; Fuchs, H. Force spectroscopy of molecular systems-single molecule spectroscopy of polymers and biomolecules. *Angew. Chem. Int. Ed.* 2000, 39(18), 3212–3237.
- 11 Pang, X.; Cheng, B.; Cui, S. The solvent quality of water for poly(*N*-isopropylacrylamide) in the collapsed state: implications from single-molecule studies. *Chinese J. Polym. Sci.* 2016, 34(5), 578–584.
- 12 Cheng, B.; Wu, S.; Liu, S.; Rodriguez-Aliaga, P.; Yu, J.; Cui, S. Protein denaturation at a single-molecule level: the effect of nonpolar environments and its implications on the unfolding mechanism by proteases. *Nanoscale* 2015, 7(7), 2970–2977.
- 13 Improt, S.; Politou, A. S.; Pastore, A. Immunoglobulin-like modules from titin I-band: extensible components of muscle elasticity. *Structure* 1996, 4(3), 323–337.
- 14 Lu, H.; Schulten, K. The key event in force-induced unfolding of titin's immunoglobulin domains. *Biophys. J.* 2000, 79(1), 51–65.
- 15 Li, H.; Oberhauser, A. F.; Fowler, S. B.; Clarke, J.; Fernandez, J. M. Atomic force microscopy reveals the mechanical design of a modular protein. *Proc. Natl. Acad. Sci. U. S. A.* 2000, 97(12), 6527–6531.
- 16 Carrion-Vazquez, M.; Oberhauser, A. F.; Fowler, S. B.; Marszalek, P. E.; Broedel, S. E.; Clarke, J.; Fernandez, J. M. Mechanical and chemical unfolding of a single protein: a comparison. *Proc. Natl. Acad. Sci. U. S. A.* 1999, 96(7), 3694–3699.
- 17 Marszalek, P. E.; Lu, H.; Li, H. B.; Carrion-Vazquez, M.; Oberhauser, A. F.; Schulten, K.; Fernandez, J. M. Mechanical unfolding intermediates in titin modules. *Nature* 1999, 402(6757), 100–103.
- 18 Rief, M.; Gautel, M.; Oesterhelt, F.; Fernandez, J. M.; Gaub, H. E. Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science* 1997, 276(5315), 1109–1112.

- 19 Florin, E. L.; Rief, M.; Lehmann, H.; Ludwig, M.; Dornmair, C.; Moy, V. T.; Gaub, H. E. Sensing specific molecular-interactions with the atomic-force microscope. *Biosens. Bioelectron.* 1995, 10(9-10), 895–901.
- 20 Zhang, W.; Zhang, X. Single molecule mechanochemistry of macromolecules. *Prog. Polym. Sci.* 2003, 28(8), 1271–1295.
- 21 Wan, Z.; Li, L.; Cui, S. Capturing the portrait of isolated individual natural cellulose molecules. *Biopolymers* 2008, 89, 1170–1173.
- 22 Cui, S.; Pang, X.; Zhang, S.; Yu, Y.; Ma, H.; Zhang, X. Unexpected temperature-dependent single chain mechanics of poly(*N*-isopropyl-acrylamide) in water. *Langmuir* 2012, 28(11), 5151–5157.
- 23 Cui, S. X.; Albrecht, C.; Kuhner, F.; Gaub, H. E. Weakly bound water molecules shorten single-stranded DNA. *J. Am. Chem. Soc.* 2006, 128(20), 6636–6639.
- 24 Cheung, M. S.; Garcia, A. E.; Onuchic, J. N. Protein folding mediated by solvation: water expulsion and formation of the hydrophobic core occur after the structural collapse. *Proc. Natl. Acad. Sci. U. S. A.* 2002, 99(2), 685–690.
- 25 Head-Gordon, T.; Brown, S. Minimalist models for protein folding and design. *Curr. Opin. Struc. Biol.* 2003, 13(2), 160–167.
- 26 Kellermayer, M. S. Z.; Smith, S. B.; Granzier, H. L.; Bustamante, C. Folding-unfolding transitions in single titin molecules characterized with laser tweezers. *Science* 1997, 276(5315), 1112–1116.
- 27 Luo, Z.; Zhang, A.; Chen, Y.; Shen, Z.; Cui, S. How big is big enough? Effect of length and shape of side chains on the single-chain enthalpic elasticity of a macromolecule. *Macromolecules* 2016, 49(9), 3559–3565.
- 28 Lum, K.; Chandler, D.; Weeks, J. D. Hydrophobicity at small and large length scales. *J. Phys. Chem. B* 1999, 103(22), 4570–4577.
- 29 Tanford, C. Protein denaturation. Part C. Theoretical models for the mechanism of denaturation. *Adv. Prot. Chem.* 1970, 24, 1–95.
- 30 Kharakoz, D. P. Partial volumes and compressibilities of extended polypeptide chains in aqueous solution: additivity scheme and implication of protein unfolding at normal and high pressure. *Biochemistry* 1997, 36(33), 10276–10285.
- 31 Kumar, A.; Venkatesu, P. Overview of the stability of α -chymotrypsin in different solvent media. *Chem. Rev.* 2012, 112(7), 4283–4307.
- 32 Larsericsdotter, H.; Oscarsson, S.; Buijs, J. Thermodynamic analysis of lysozyme adsorbed to silica. *J. Colloid Interface Sci.* 2004, 276(2), 261–268.
- 33 Dietz, H.; Rief, M. Exploring the energy landscape of GFP by single-molecule mechanical experiments. *Proc. Natl. Acad. Sci. U. S. A.* 2004, 101(46), 16192–16197.
- 34 Alexander, P.; Fahnstock, S.; Lee, T.; Orban, J.; Bryan, P. Thermodynamic analysis of the folding of the streptococcal protein G IgG-binding domains B1 and B2: why small proteins tend to have high denaturation temperatures. *Biochemistry* 1992, 31(14), 3597–3603.
- 35 Liu, C.; Cui, S.; Wang, Z.; Zhang, X. Single-chain mechanical property of poly(*N*-vinyl-2-pyrrolidone) and interaction with small molecules. *J. Phys. Chem. B* 2005, 109(31), 14807–14812.
- 36 Oesterhelt, F.; Rief, M.; Gaub, H. E. Single molecule force spectroscopy by AFM indicates helical structure of poly(ethylene-glycol) in water. *New J. Phys.* 1999, 1, 6.1–6.11.
- 37 Cui, S. Single-molecule force spectroscopy of biomacromolecules: comparative studies in aqueous solution and nonpolar solvents. *Acta Polymerica Sinica (in Chinese)* 2016, (9), 1160–1165.