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Single-molecule force-unfolding of titin I27 reveals correlation between size of surrounding anions and its mechanical stability

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Each cellular protein is surrounded by a biochemical milieu that affects its stability and the associated function. The role of this surrounding milieu in proteins' mechanical stability remains largely unexplored. Herein we report as yet unknown correlation between the size of surrounding anion and the mechanical stability of protein. Using single-molecule force spectroscopy of the 27th domain (I27) of human cardiac muscle protein titin, we show that the average unfolding force of the protein decreases with increase in the ionic radii of the surrounding anions in the order Cl^- > Br^- > NO_3^- > I^- > SO_4^{2-} ≈ ClO_4^- indicating an inverse correlation between anion size and mechanical stability of I27. The destabilizing effect was attributed to the combined effect of increase in unfolding rate constant and unfolding distance upon incubation of the anion. These findings reveal that anion size can significantly affect mechanical resistance of proteins and thus could be a convenient and promising tool for regulating mechanical stability of proteins.

Protein-based nanomaterial's have a great potential in biomedical applications owing to diversity in their mechanical properties, relative ease of modifications and production of proteins, and biocompatibility. For their use in nanomaterial's, it is desirable that protein's mechanical properties such as mechanical stability could be precisely fine-tuned for specific applications, and thus, there have been enormous efforts to understand the basis of mechanical properties [1, 2]. As protein folds, the protein backbone and amino acid side chains interact with the surrounding medium, which influences the protein's thermodynamic and mechanical stability [3-5]. The understanding of how the immediate environment affects the protein's mechanical stability offers a great advantage in designing strategies to modulate mechanical stability of proteins.

The salt ions present in the surrounding milieu play crucial role in determining protein stability and associated biological activity. Salts are also generally used to modulate osmolarity of solutions for various specific applications and accordingly have been examined for their effect on protein properties. It is known that salt ions affect thermodynamic stability of the protein. At lower concentrations (<0.35 M), the salt effect is mediated by screening of coulombic interactions, whereas at high concentrations, they follow the Hofmeister series, which for anions, follow the order PO_4^{3-}> SO_4^{2-}> HPO_4^{2-}> F^-> Cl^-> NO_3^-> Br^-> I^-> ClO_4^- [6-8]. Although the effect of salt ions on thermodynamic stability is extensively investigated, not much is known about their influence on mechanical stability of proteins, which is primarily kinetic stability along the mechanical unfolding pathway.

Atomic force microscopy (AFM)-based single-molecule force spectroscopy (SMFS) has emerged as a powerful approach to exploring mechanical properties of proteins. These single-molecule studies have revealed various novel properties of proteins that have previously not been accessible by other traditional ensemble methods[9-16]. It has been shown that unlike thermodynamic stability, mechanical resistance to protein unfolding primarily depends upon the topology of the force-bearing region of a protein[16, 17]. Studies indicate that the force-bearing region of proteins with significant mechanical stability is composed of parallel β-strands connected through a network of hydrogen bonds that acts as a clamp against an applied force[16, 18]. Similarly, other studies have examined the contribution of hydrophobic and electrostatic interactions to mechanical stability of proteins [19, 20, 21-23 24].

In the present study, we carried out SMFS of the 27th (I27) domain of human cardiac protein titin to understand how various salts of different ion size and charge affect this protein's mechanical stability.

We characterized the unfolding behavior of the I27 polyprotein in solution with anions of different sizes at the same pH. The study reveals previously unknown dependence of mechanical stability of proteins on the size of salt ions. Our study provides a new way to fine-tune mechanical stability of a protein and holds promise for the design of protein-based novel biomaterials for biomedical and materials science applications.

We first examined the force–extension relation of polyprotein $(I27)_8$ in phosphate buffer (PB) at 400 nm·s^{-1}. A schematic of the sequence of events during single-molecule pulling experiments using atomic force spectroscope is given in SI, Fig. S1. Fig. 1A shows force–extension profiles containing a sawtooth-like pattern, which is characteristic of unfolding of a single polyprotein molecule attached to a cantilever tip and to a surface. Each consecutive peak was fitted to the wormlike chain model of polymer elasticity providing a contour length increment of ~27.6 nm, which is in good agreement with other studies[17](SI, Fig. S2). The average unfolding force of $(I27)_8$ in phosphate buffer obtained by fitting the force histogram to a

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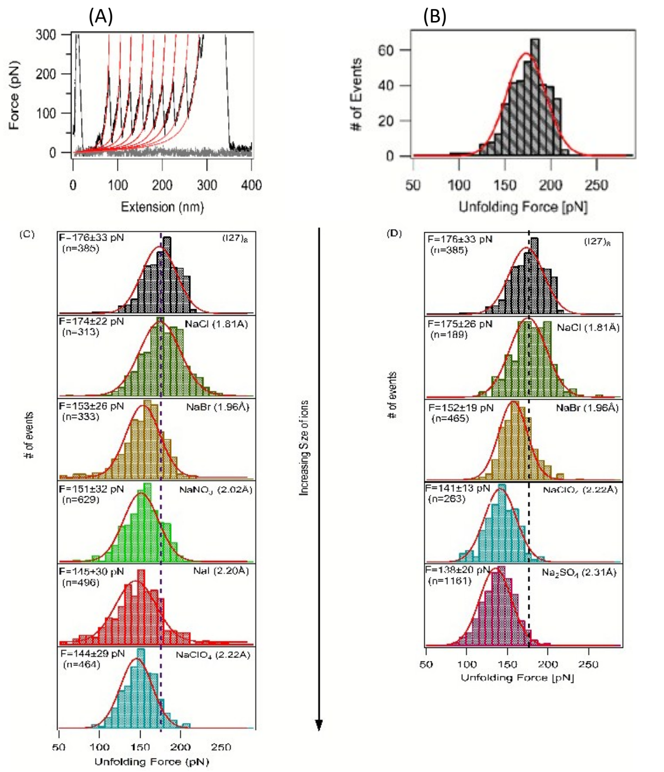

Fig. 1: (A) A representative force−extension curve of $(I27)_8$ in PB. Red lines correspond to wormlike chain (WLC) fit. (B) An unfolding force frequency histogram of $(I27)_8$ at a pulling speed of 400 nm/s in PB. The Gaussian fit (red curve) to the histogram yields an unfolding force of 176 ± 33 pN (n = 385). (C) An unfolding force frequency histogram for $(I27)_8$ stretched in PB in the presence or absence of different anions (1M). The solid line denotes a Gaussian fit to the histogram. As seen, the average unfolding force of I27 decreased with an increase in anion size. (D) A force histogram of the mechanical unfolding force for $(I27)_8$ in PB alone or in the presence of 0.2 M NaCl, NaBr, $NaClO_4$ or Na_2SO_4. The force histogram for the PB group is reproduced from that in panel (A).

Gaussian distribution was found to be 176±33pN (Fig. 1B) [11, 25-27] To evaluate the role played by anion size in mechanical properties of proteins, we investigated the salt effect on mechanical stability of I27 by choosing a number of anions (as a sodium salt) carrying the same charge but different ionic radius [Cl^- (1.81 Å), Br^- (1.96 Å), NO_3^- (2.02 Å), I^- (2.20 Å) and ClO_4^-(2.22 Å)][28] (SI, Table S1). The use of similarly charged anions rules out the charge effect and exclusively enables selective detection of anion size–dependent effects on the mechanical stability of the protein. The I27 polyprotein was preincubated with various anions in PB while Na^+ served as a common cation for pulling experiments. The representative force−extension curves of $(I27)_8$ in absence and presence of different anions is shown in SI, Fig. S3. We first measured the mechanical stability of $(I27)_8$ in the presence of 1M Cl^-, which is an anion of intermediate size as per Hofmeister series. As depicted in Fig. 1C, average unfolding force in ~1.0 M chloride was found to be 174 ± 22 pN, which is similar to that seen in PB (176 pN), suggesting that chloride ion has no significant effect on the mechanical stability of I27. Next, stretching experiments were performed on I27 preincubated with ~1.0 M bromide, an anion of a relatively bigger size, in place of Cl^-. Analysis of the resulting force histograms yielded an average unfolding force of 153 ± 26 pN for I27 incubated with 1.0 M bromide. Clearly, relative to chloride ion, the average unfolding force in the presence of 1.0 M bromide decreased by ~20pN, suggesting, the presence of bromide has a destabilizing effect on the mechanical stability of I27.

To further examine whether the destabilizing effect of Br^- is associated with its relatively greater anion size, we next carried out single-molecule pulling experiments in the presence of 1.0 M NO_3^-, which has a size (2.02 Å) nearly similar to that of Br^-. Analysis of a force histogram obtained from more than 150 events revealed that the average unfolding force for I27 in the presence of 1 M NO_3^- is 151 ±32 pN, which is similar to that observed in the presence of Br^-, implying that the decrease in mechanical stability after incubation of the protein with Br^- or NO_3^- could be related to their anion size.

To further elucidate the relation between anion size and mechanical stability of the protein, we examined force-dependent unfolding of the I27 polyprotein in the presence of anions of relatively larger size in comparison with bromide in the Hofmeister series. The iodide (2.20 Å) anion is ~0.20 Å bigger than bromide and nitrate. The single-molecule pulling experiments in the presence of 1.0 M iodide showed that the mechanical stability of I27 decreases further to 145 pN as compared to 153 pN in the presence of bromide and 176 pN in PB alone. We next examined mechanical unfolding force in the presence of ClO_4^- (2.22 Å), i.e., an anion of a size similar to that of I^-. The results showed that average unfolding force in the presence of ClO_4^- (2.22 Å) remains similar to that obtained in the presence of I^- (144 versus 145 pN).

As the mechanism by which anions affect protein stability varies with anion concentration, we next determined whether the observed effect on mechanical stability holds true even at lower concentrations (Fig. 1D). Because I27 mechanical stability was found to be similar in 1.0 M of Br^- and NO_3^- solutions, as was the case for I^- and ClO_4^-, the unfolding force at a lower concentration was examined only in the presence of 0.2 M Cl^-, Br^-, or ClO_4^- as representative anions. Fig. 1D shows a force histogram obtained from stretching the I27 polyprotein, a single molecule at a time, in the presence of Cl^-, Br^-,or ClO_4^-. Of note, even at a lower anion concentration of 0.2 M, the mechanical stability of I27 polyprotein was found to decrease from 176 pN to 152 pN in the presence of Br^- or 141 pN in the presence of ClO_4^-, which were of magnitude similar to that seen in the presence of 1.0 M anion concentration (152 versus 153 pN for Br^- and 141 versus 144 pN for ClO_4^-). The data suggested that the negative effect of low-charge-density anions on mechanical stability reaches saturation at a concentration of 0.2 M or below.

Above results show the effect of anions, generally known as chaotropes on the mechanical stability of protein. If the effect of the above anions is related to their ionic radii, then even kosmotropes of similar size (which are generally known to stabilize a protein) should also have a negative effect on the mechanical stability of the protein. We thus examined the effect of a kosmotrope SO_4^{2-}, of size 2.31 Å, on mechanical stability of $(I27)_8$. The protein was preincubated with either 1 M or 0.2 M SO_4^{2-} however due to visible aggregation of the $(I27)_8$ polyprotein in the presence of 1 M SO_4^{2-}, the force spectroscopy experiments could be performed only at the lower concentration (0.2 M) of the salt solution. Similar to as seen above for other anions of similar size, a decrease in mechanical stability of I27 was observed in the presence of SO_4^{2-} (Fig. 1D).

The above results indicate that anion size influences the mechanical stability of I27. We next tested whether the anion-mediated decrease in mechanical stability is reversible. To examine the reversibility, we used sulfate or perchlorate generally known as a kosmotropic or chaotropic anion, respectively. The protein was incubated with 1.0 M perchlorate or 0.2 M sulfate for 6 h.

The salt ions were removed by extensively dialyzing the protein against PB. The pulling experiments with pre- and post dialyzed protein samples were conducted with the same cantilever to avoid any

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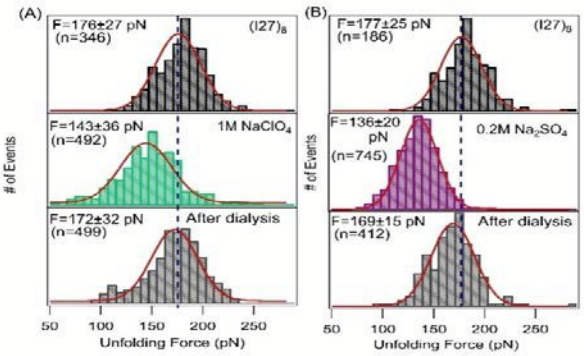

Fig. 2: The decrease in mechanical stability by anions is reversible. The protein in PB was preincubated with (A) 1M $NaClO_4$ or (B) 0.2 M Na_2SO_4. The protein solution with added salt ions was extensively dialyzed against PB for examining reversibility of the force change induced by salt ions. Shown is the force histogram obtained with I27 in PB (upper panel), PB with added salt ions (middle panel), and a solution dialyzed extensively against PB (lower panel). All experiments with $NaClO_4$ or Na_2SO_4 were conducted with the same cantilever at a pulling speed of 400 nm·s^{-1}. The Gaussian fit of the force histogram is presented as a solid brown curve.

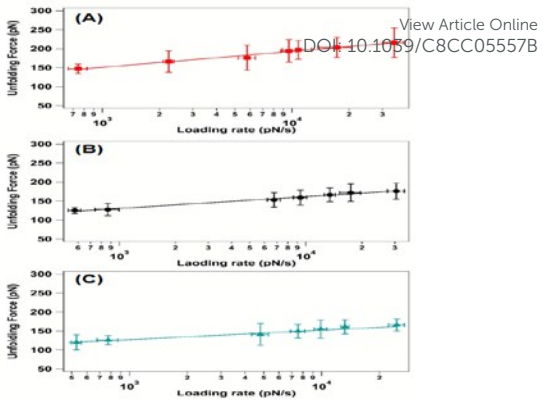

Fig. 3: Loading-rate dependence of the unfolding force of $(I27)_8$ in PB (A) in the absence (■) or presence (●) of 0.2 M NaBr (B) or (▲) 1M $NaClO_4$ (C). The single polyprotein molecule was stretched at different pulling speeds. The symbols correspond to the average of the force obtained at a single pulling speed, and the experimental data were fitted to the Bell–Evans model.

bias from the cantilever. As shown in Fig. 2, the presence of perchlorate and sulfate decreased the mechanical stability by 33 and 41 pN, respectively. The removal of perchlorate and sulfate by dialysis restored the mechanical stability of I27 to 172 pN and 169 pN, respectively, suggesting that the destabilizing effect of these anions on mechanical stability is reversible in nature.

To further examine the reversibility of mechanical stability upon replacing one salt ion with another, we carried in situ single molecule pulling experiment with I27 in the presence of NaCl or $NaClO_4$ (Fig. S4). The in situ study allows more controlled conditions with less variability and thus important to remove any biasness from the use of different cantilevers and other experimental conditions. The $(I27)_8$ in PB was adsorbed onto the coverslip. The NaCl was then added to a final concentration of 1M. The protein was incubated with buffer for 30 min and unfolding force was measured. Buffer containing NaCl was then replaced with 1M $NaClO_4$ in situ. The force extension curves were recorded, and then $NaClO_4$ was further replaced with buffer containing 1M NaCl to monitor its effect on unfolding force. As shown, $NaClO_4$ decreased the mechanical stability of I27 which was further restored as the salt was replaced by NaCl.

To examine the effect of anions on kinetic parameters underlying the free-energy landscape of I27, the polyprotein was stretched at pulling speed varying from 100 to 6400 nm/sec in the presence or absence of 0.2 M Br^- (Fig. 3). As expected, the unfolding force, irrespective of the presence or absence of anions, was found to be dependent upon pulling rates, such that the unfolding force increased logarithmically with an increase in the pulling rate. The pulling-rate dependence was fitted well to the Bell–Evans model [29] [30]. Fig. 3A, shows the best fit of the data to the Bell–Evans model with the spontaneous unfolding rate constant (α_0) of 3.0×10^{-4} and 5.5×10^{-4} s^{-1} in the absence and presence of bromide, respectively. Assuming a pre-exponential factor of 10^9 s^{-1}, the mechanical unfolding barrier was found to be 71 kJ/mol (17 kcal/mol) and 70 kJ/mol (16.7 kcal/mol) in the absence and presence of Br^-, respectively, suggesting that the transition state energy barrier of mechanical unfolding is marginally lowered (by ~1kJ/mol) in the presence of the anion. The unfolding distance (Δx_u) was found to be 0.25 and 0.28 nm in the absence and presence of bromide, respectively, implying that the presence of the anion moves the transition state closer to the denatured state[16, 26] (Table 1). Similar t as seen in the presence of Br^-, an increase in Δx_u was also observed upon stretching I27 in the presence of 1M ClO_4^- (Table 1).

We further carried out equilibrium urea denaturation experiments to determine the thermodynamic stability of monomeric I27 (I27-M) in the presence and absence of salts (0.2M NaBr or 1M $NaClO_4$) as described in Materials and Methods. As shown in Fig. S5 the protein unfolds in a two state manner. The fit of denaturation curve to two state model provides $\Delta G_{N\text{-}D}$ of 7.4 kcalmol^{-1}, 6.6 kcalmol^{-1} and 5.1 kcalmol^{-1} in PB, PB with 0.2M NaBr and PB with 1M $NaClO_4$ respectively suggesting that the salt ions affect the stability of the native proteins (SI, Table S2). Fig. S6 (SI) shows the mechanical unfolding free energy profile of I27 in the presence of PB alone or with 0.2M NaBr or 1M $NaClO_4$.

SMD simulation is widely used to gain insights into the mechanism underlying the mechanical stability of proteins[31, 32]. Due to computational cost, five SMD simulations were performed for each pulling velocity of 0.5 Å/ps and 0.1 Å/ps, with a harmonic constraint force of 10.0 kcal/(mol·Å^2) in different solvated systems, water, 1 M NaCl, or 1 M NaI, to compare the force-induced unfolding of I27. Fig. S7 (SI) provides comparison of I27 structure obtained from SMD simulations before and after the main burst phase. The Force vs Extension profile obtained from one of the N-terminal pulling experiments in water or NaCl or NaI in a 0.5Å/ps SMD simulation is shown in Fig.4. Peak values for the applied forces in 1M Nacl or 1M NaI were ~2157.60 and ~2012.98 pN, respectively for the constant velocity N-terminal pulling at 0.1 Å/ps, whereas in case of 0.5 Å/ps pulling velocity, the peak values were ~3473.28 and ~3331.52 pN, respectively (SI, Table S3). The SMD simulations thus show a relatively lower force peak value in NaI as compared to NaCl.

The present study reveal an unexpected relation between anion size and the mechanical stability of I27. Most ions are hydrated in water however the hydrated radii of the ions does not vary much with change in their bare ionic radii e.g. the hydrate radii of Cl^-, I^- and ClO_4^-

Table 1: Mechanical properties of $(I27)_8$ in the presence or absence of 0.2M NaBr or 1M $NaClO_4$

Buffer	Unfolding Force(pN)	ΔL_c(nm)	Δx_u(nm)	α_0 (s^{-1})	$\Delta G^{\#}_{N\text{-}T}$ (kJ/mol)
PB	176 ±33	27.6	0.25	3.0×10^{-4}	71
PB + NaBr	152 ±19	27.4	0.28	5.5×10^{-4}	70
PB + $NaClO_4$	144 ±29	27.4	0.30	7.0×10^{-4}	69

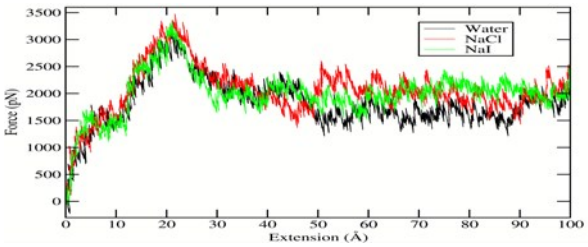

Fig. 4: Constant-velocity SMD simulations of I27. Mechanical pulling of I27 in water as a solvent in the presence and absence of NaCl or NaI using constant-velocity SMD simulations. The force extension curves of I27 were obtained at a pulling velocity of 0.5 Å/ps.

is 3.32 Å, 3.31 Å and 3.38 Å respectively [33] [34]. Thus the observed correlation of mechanical stability is with respect to the thermochemical radii of anions. As the mechanical stability varies with the size of the salt ions, the effect could not be merely attributed to the increase on ionic strength of the solution. Similarly other ionic properties such as dipole moment, polarizbility did not show much correlation with the mechanical stability (SI, Table S1). Also as ΔL_c of I27 with different anions remains similar, the protein's native structure is not significantly altered after addition of these anions (SI, Fig. S2). Thus, the anion-mediated effect on mechanical stability is more specific and could be due to the influence of anions on the interactions required for resisting unfolding forces.

Several lines of evidence revealed that the anion size–mediated decrease in mechanical stability of the I27 polyprotein is due to ionic screening of electrostatic interactions. First, it is expected that if an anion size–induced decrease in mechanical stability of I27 is due to interference with hydrophobic interactions, then sulfate would increase the mechanical stability of the protein by strengthening intramolecular hydrophobic interactions. Second, at anion concentrations of 0.2 M, the effect on mechanical stability was primarily dominated by ionic screening and not hydrophobic effects, which become predominant at a relatively higher concentration (≥0.35 M). As shown in Fig. 1 (C, D), the anion-induced decrease in the average unfolding force is similar for ~0.2 M and 1.0 M anions. These findings argue against any hydrophobic effect of anions as a driving force behind the anion-induced decrease in mechanical stability of the protein. Taken together, these results reveal that the anion size–mediated decrease in mechanical stability of the I27 polyprotein derives from ionic screening of electrostatic interactions. The oxidation of protein is also known to affect the mechanical stability of the protein and the effect is primarily irreversible [35]. As the effect on mechanical stability observed in the present study is reversible the underlying basis of decrease in mechanical stability is independent of any oxidative effect of the anions. The anion size-dependent mechanical stability revealed in the present study provides a new understanding of the mechanical unfolding of proteins and offer a unique approach to fine-tuning mechanical stability of proteins to achieve desirable mechanical properties.

Conflicts of interest: There are no conflicts to declare.

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