



Interaction of chloramphenicol with titin I27 probed using single-molecule force spectroscopy

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Abstract

Titin is a giant elastic protein which is responsible for passive muscle stiffness when muscle sarcomeres are stretched. Chloramphenicol, besides being a broad-spectrum antibiotic, also acts as a muscle relaxant. Therefore, it is important to study the interaction between titin I27 and chloramphenicol. We investigated the interaction of chloramphenicol with octamer of titin I27 using single-molecule force spectroscopy and fluorescence spectroscopy. The fluorescence data indicated that binding of chloramphenicol with I27 results in fluorescence quenching. Furthermore, it is observed that chloramphenicol binds to I27 at a particular concentration ($\sim 40 \mu\text{M}$). Single-molecule force spectroscopy shows that, in the presence of $40 \mu\text{M}$ chloramphenicol concentration, the I27 monomers become mechanically stable, resulting in an increment of the unfolding force. The stability was further confirmed by chemical denaturation experiments on monomers of I27, which corroborate the evidence for enhanced mechanical stability at $40 \mu\text{M}$ drug concentration. The free energy of stabilization for I27 (wild type) was found to be 1.95 ± 0.93 kcal/mole and I27 with $40 \mu\text{M}$ drug was 3.25 ± 0.63 kcal/mole. The results show a direct effect of the broad-spectrum antibiotic chloramphenicol on the passive elasticity of muscle protein titin. The I27 is stabilized both mechanically and chemically by chloramphenicol.

Keywords Titin I27 · Chloramphenicol · Single molecule force spectroscopy · AFM

1 Introduction

The binding of small molecules such as drugs and dyes to biological macromolecules is active area of investigation [1]. It is important to understand the efficacy as well as side effects of some broad-spectrum drugs such as chloramphenicol (CLM) and aureomycin [2, 3]. This investigation of protein-drug interaction is important for pharmacology and

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pharmacokinetics [4]. Each drug has a therapeutic window for treatment of various diseases. For instance, it has been shown that the inhibitory concentration of CLM for various organisms is 4–12.5 mg/L (12–38 μ M). The peak serum concentration for CLM is in the range of 15–25 mg/L (46–77 μ M) and the trough concentration is found to be around 5–10 mg/L (15–30 μ M) [5]. The toxicity is reported to increase when concentration rises above 25 mg/L [6]. This demands a proper understanding of drug-protein interaction at various concentrations and how concentration of drug molecules affects the functioning of a protein.

Protein-drug interaction can be studied by direct or non-separation methods such as ultraviolet-visible absorption spectroscopy and fluorescence spectroscopy and indirect techniques or separation methods such as gel filtration, ultracentrifugation, and equilibrium dialysis [7–9]. It is also extensively investigated using fluorescence quenching [10–13]. Recently, there are reports of the effect of a drug on the mechanical properties of single molecules of proteins [14]. Single-molecule techniques wherein a force is applied on single molecule to produce mechanical unfolding can be used to investigate the drug-protein interaction at the level of single molecules [15]. In this work, we report the effects of CLM drug on elasticity of I27, one of the immunoglobulin (Ig) domains of muscle protein titin using atomic force microscopy (AFM).

Titin is a giant muscle protein and contains two types of domains. One of them is fibronectin type (III) and the other domains are immunoglobulin (Ig) [16]. Furthermore, these domains are arranged in an array of N-terminal I-band consisting of Ig domains and C-terminal A-band that consists of alternating fibronectin and Ig domains. Titin is also known as connectin and acts as a molecular spring which is responsible for passive force (elasticity) in muscle sarcomere and stability of myosin filaments [17]. The mechanical response of the single octamer of I27, one of the Ig domains from the I-band is measured using AFM [18, 19]. The interaction of titin I27 with calcium and its effect on the mechanical properties were investigated using fluorescence and AFM [20].

CLM is a broad spectrum antibiotic which is used for treatment of bacterial infections and it is also a muscle relaxant. It is known to have a direct impact on muscles which is concentration-dependent [21]. The myocardial effects of CLM on rat heart muscle have been investigated and are reported to cause depressed isometric contraction [22].

The effect of certain antibiotics such as CLM, kanamycin, and streptomycin was investigated on rat heart muscles and was shown to have a direct negative inotropic effect on isolated rat heart muscles [23].

Chick embryos were found to have a significantly reduced heart rate at large concentrations (250 μ g/ml) of CLM [24]. This has led to reports on interaction of CLM with many proteins such as human serum albumin (HSA) [25, 26] and lysozyme [27]. However, there is no understanding if CLM binds to titin, and its effect on elasticity of titin which is a major part of the muscle sarcomere.

In this work, we first investigated protein-drug interaction in the bulk using fluorescence quenching. We found that fluorescence intensity decreases with an increase in the concentration of the drug, providing evidence of CLM binding to I27. Furthermore, using single-molecule force spectroscopy, we unraveled the effect of drug on mechanical stability of I27 polypeptide at various concentrations. We found that the unfolding force increases when CLM binds. It is maximum at a concentration of 40 μ M. The increase in unfold-

ing force at this concentration is ~ 25 pN. The entropic stiffness of the unfolded chain is also increased due to this binding. The mechanical stability seen at $40 \mu\text{M}$ is also seen in equilibrium chemical denaturation experiments which were performed with monomers of I27.

2 Methods

2.1 Protein expression and purification of I27 monomer (I27)₁ and I27 octamer (I27)₈

Plasmid pET-23a containing the human I27 gene with an ampicillin resistance marker was transformed into *Escherichia coli* BL21(DE3) chemical competent cells. One isolated colony was selected and grown in LB medium at 37°C until OD600 reached 0.6. Once OD600 reached 0.6, then the culture was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and incubated at 37°C , 180 rpm for 6 h. The cells thus obtained were lysed with lysis buffer containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.25% β -mercaptoethanol using ultrasonication. The clarified lysate obtained after cell lysis was loaded on a pre-equilibrated 5 mL HiPrep Ni-NTA affinity column (GE Healthcare). The composition of equilibration buffer was 50 mM Tris-HCl pH 8.0 and 300 mM NaCl. Finally, the protein was eluted with an equilibration buffer containing 250 mM imidazole. Fractions containing desired proteins were checked on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and pooled together. Imidazole was removed by buffer exchange (10 mM PBS pH 7.4) using amicon tubes (Merck, MWCO: 10 kDa). Protein was flash frozen in liquid nitrogen with 10% glycerol and stored at -80°C for further use. The concentration of protein was checked on nanodrop each time before the experiment. The I27 octamer was also purified using a similar procedure.

2.2 Fluorescence spectra

A total of 1 mM stock solution of CLM was prepared in 10 mM PBS pH 7.4. I27 monomer was dialyzed in 10 mM PBS pH 7.4 for 10–12 h. Samples of different concentrations of CLM (0, 10, 20, 30, 40, 50, 75, and $100 \mu\text{M}$) with a constant protein concentration of $40 \mu\text{M}$ were made in 10 mM PBS pH 7.4. The total volume of each solution was made up to $100 \mu\text{L}$ with 10 mM PBS buffer pH 7.4 only. Fluorescence measurements were performed 96-well plates at room temperature with excitation wavelength, λ_{ex} : 295 nm and emission scan range between 315 and 600 nm. All the fluorescence measurements were done on a Perkin Elmer Enspire multimode reader.

2.3 Docking studies

The available crystal structure of I27 (PDB ID: 1TIT) was docked with CLM using the PatchDock server [28]. PatchDock is an online molecular docking method that predicts the structure of protein and small-molecule complex based on shape complementarity. Out of the ten models provided by the server, we chose the best fit model considering the docking score. Chimera was used for graphical analysis and figure generation.

2.4 Atomic force spectroscopy

An atomic force microscope is used to perform the force-extension measurements on the polyprotein of I27. The protein is tethered between a tip on microcantilever and substrate. The cantilevers were calibrated for the force constant before each experiment. When the relative distance of cantilever-substrate is changed with a constant speed along the perpendicular direction to the substrate, domains sequentially unfold (Fig. 1). Each peak in the pattern represents the force required to unfold a domain which can be associated with the mechanical stability of the domain along the direction of pulling.

For protein-drug sample preparation, an appropriate amount of drug was added to the protein sample. The sample was mixed properly and then incubated on a gold-coated coverslip for 30 min. While for (I27)₈ without drug, only I27 protein (2–5 μM , 40 μL) was incubated on a gold-coated coverslip for 30 min. After incubation, the sample was washed

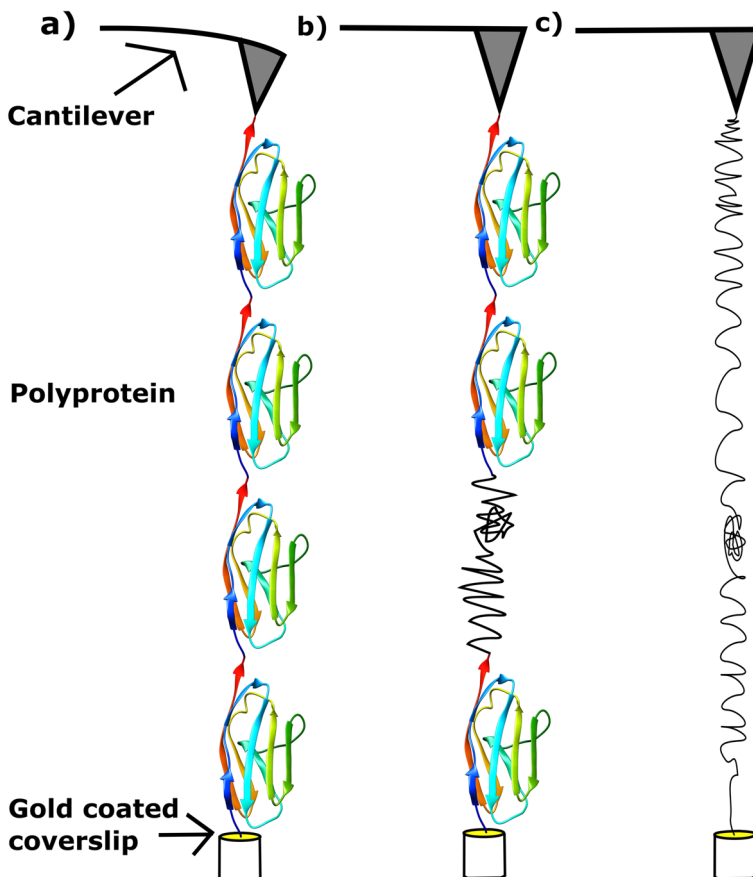


Fig. 1 **a** Schematic of I27 octamer tethered between AFM cantilever and gold-coated coverslip. **b** One of the domains becomes unfolded when the applied force reaches a critical value, resulting in relaxation of the cantilever to its equilibrium position. **c** Unfolding of last domain. Further pulling will result in the stretching of the unfolded domain followed by tip-molecule detachment

with 10 mM PBS pH 7.4 to remove unbound protein. After 2–3 washings, 600 μ L of PBS pH 7.4 was taken in a liquid cell and force spectroscopy was performed.

The experiments were performed in a commercial AFM (JPK Nanowizard-II, Berlin, Germany). Rectangular silicon-nitride cantilevers from Micromasch (Bulgaria) with stiffness ~ 0.05 N/m were used for the measurements. Before every measurement, the cantilever was calibrated at room temperature in buffer using thermal noise method.

2.5 Equilibrium denaturation of (I27)₁

Three different sets of experiments with varying concentrations of CLM were performed. In one set of experiment, the concentrations of protein (40 μ M) and CLM (30 μ M) were kept constant while the concentration of guanidinium hydrochloride (GdmCl) was varied. All the samples with varying GdmCl concentration were gently mixed and incubated overnight at 28 °C in a water bath. The same protocol was followed for the rest of two different concentrations of CLM, 40 and 50 μ M. Fluorescence measurement was done on 96-well plates at 28 °C with excitation wavelength, λ_{ex} at 295 nm, and emission scan range between 315 and 600 nm. All the fluorescence measurements were performed on a Perkin Elmer Enspire multimode reader (IISER Pune).

3 Results

3.1 Fluorescence

3.1.1 Effect of CLM on tryptophan fluorescence quenching

Fluorescence quenching has been widely used in investigating interactions between proteins and small drug molecules. The change of the microenvironment around a fluorophore changes the fluorescence intensity. Fluorescence quenching in a system is a result of energy transfer or complex formation between the two molecules. The fluorescence quenching data is useful in deciphering and quantifying the protein-drug binding.

Here, we checked the binding of CLM by monitoring the tryptophan (Trp) fluorescence (λ_{ex} , 295 nm; λ_{em} , 330 nm) in I27 protein. Titin I27 has one Trp and one tyrosine (Tyr). When excited at 280 nm, we will have contribution from both Trp and Tyr, whereas when excited at 295 nm, Trp gets excited selectively. We observed that with an increase in CLM concentration, the intensity of fluorescence emission of (I27)₁ decreased without a shift in the emission maxima (330 nm). These quenching experiments suggest that CLM interacts with I27 and its binding site is close to Trp (Fig. 2).

The proximity of CLM to Trp was further confirmed by molecular docking studies. Using PatchDock, we identified the residues involved in the interaction of I27 with CLM. We found that the distance between the CLM and Trp34 in the selected model is less than 5 Å (Fig. 3). This explains the quenching of the Trp fluorescence which we observed in our previous results.

3.1.2 Mechanism of fluorescence quenching

The mechanism of quenching can be classified into two categories: static and dynamic quenching. Static quenching usually refers to the formation of a complex between the two molecules, i.e., protein (fluorophore) and drug (quencher) molecule, whereas dynamic

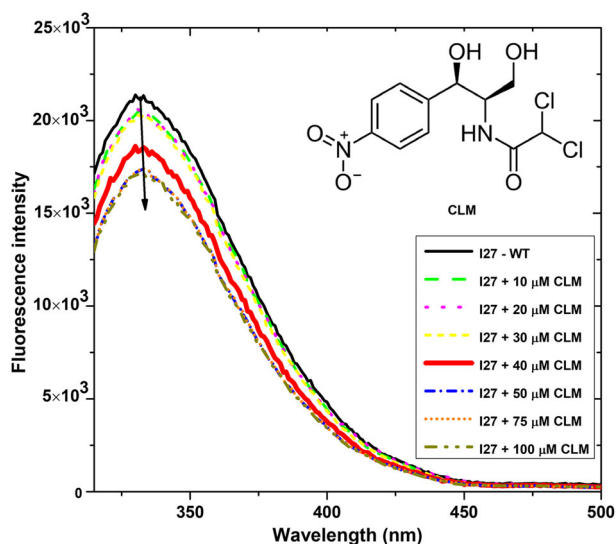


Fig. 2 The Trp fluorescence emission spectra of I27 excited with 295 nm wavelength after the addition of CLM to the solution. The arrow indicates increasing concentrations of CLM (0, 10, 20, 30, 40, 50, 75, and $100 \times 10^{-6} \text{ mol L}^{-1}$). We observed that the fluorescence is quenched with the addition of CLM to the solution. 10 mM PBS (pH 7.4) buffer is used for the protein as well as for CLM solution

quenching occurs when excited fluorophore interacts with other molecules and thereby results in non-radiative transition [29]. Generally, quenching data is analyzed using the Stern-Volmer equation

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q \tau_0 [Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, K_{SV} is the Stern-Volmer constant, k_q is the quenching rate constant of the biological molecule, τ_0 is the fluorescence lifetime in the absence of the quencher, and $[Q]$

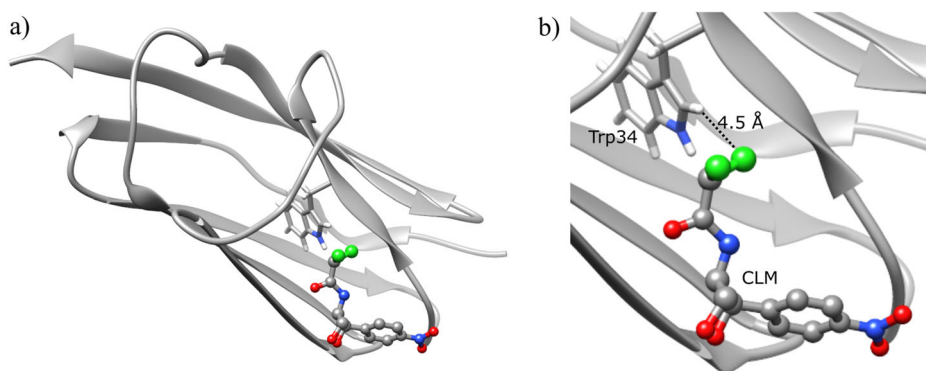


Fig. 3 Docking studies to check the proximity of CLM to Trp34 of titin I27 protein. **a** I27 (PDB ID: 1TIT) docked with CLM using PatchDock. **b** Interaction of CLM with Trp34 within 5 Å. It suggests that the fluorescence quenching is due to attachment of CLM to the protein in vicinity of Trp

is the quencher concentration. Figure 4 shows the Stern-Volmer plot for the quenching of I27 fluorescence due to binding of CLM.

In the case of static quenching, fluorescence lifetimes remain constant in the presence of the quencher ($\tau_0/\tau = 1$), unlike collisional quenching where ($\tau_0/\tau = F_0/F$). The fluorescence lifetime of the biomolecule is 10^{-8} s [30], with a maximum dynamic quenching constant k_q^{max} for various quenchers known to be 2.0×10^{10} L mol $^{-1}$ s $^{-1}$ [31]. The value of the quenching constant (K_{SV}) here is found to be 3.795×10^3 L mol $^{-1}$. This corresponds to a dynamic k_q of order 10^{11} L mol $^{-1}$ s $^{-1}$, which is larger than k_q^{max} , indicating that quenching here is predominantly static. These results demonstrate that the interaction between I27 and CLM molecules results in the formation of a complex.

3.2 Atomic force spectroscopy

Figure 5a and b show representative force curves of the unfolding of I27 without and with CLM molecule attached, respectively. We see 8 unfolding events of the octamer of I27. The unfolding force was measured for I27 and I27 in the presence of 40 μ M CLM. We observed that the force required to unfold the domains of I27-WT (wild type) is 218 ± 2 pN. On the other hand, the force needed to unfold the domain of I27 in the presence of 40 μ M CLM is 242 ± 2 pN. The unfolding force for I27 with 40 μ M CLM is 25% higher than the I27-WT. Figure 6a represents the unfolding forces of I27 at different concentrations of CLM. Figure 6b represents the persistence length for I27-WT and I27 with 40 μ M CLM. The peak number corresponds to the total number of domains which still remains folded at this particular unfolding event. For instance, for a I27-octamer, the peak number 6 specifies that there are only 2 folded domains remaining since there are a total of 8 domains in the polypeptide. The fluorescence quenching data suggests that at 40 μ M concentration, CLM attaches to the I27 near Trp. The enhancement in force required to mechanically unfold the protein in the presence of CLM with 40 μ M indicates that the CLM mechanically stabilizes titin's individual domains.

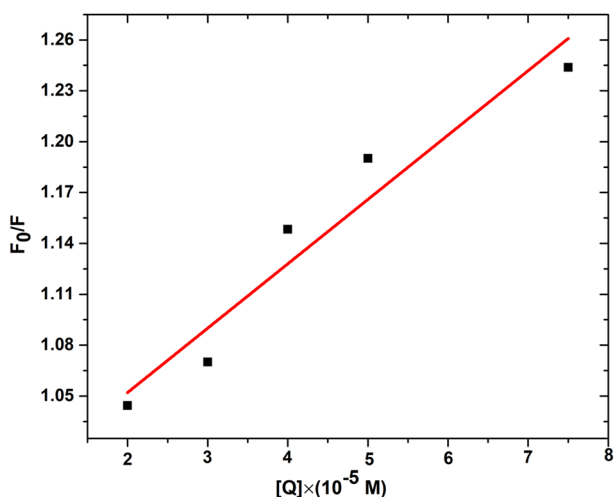


Fig. 4 Stern-Volmer plot for quenching of I27 at different drug concentrations (pH is 7.4, the excitation wavelength, λ_{ex} = 295 nm). The concentration of I27, [I27] = 40×10^{-6} M, the drug concentration is varied, [Q] = 20, 30, 40, 50, and 75×10^{-6} M

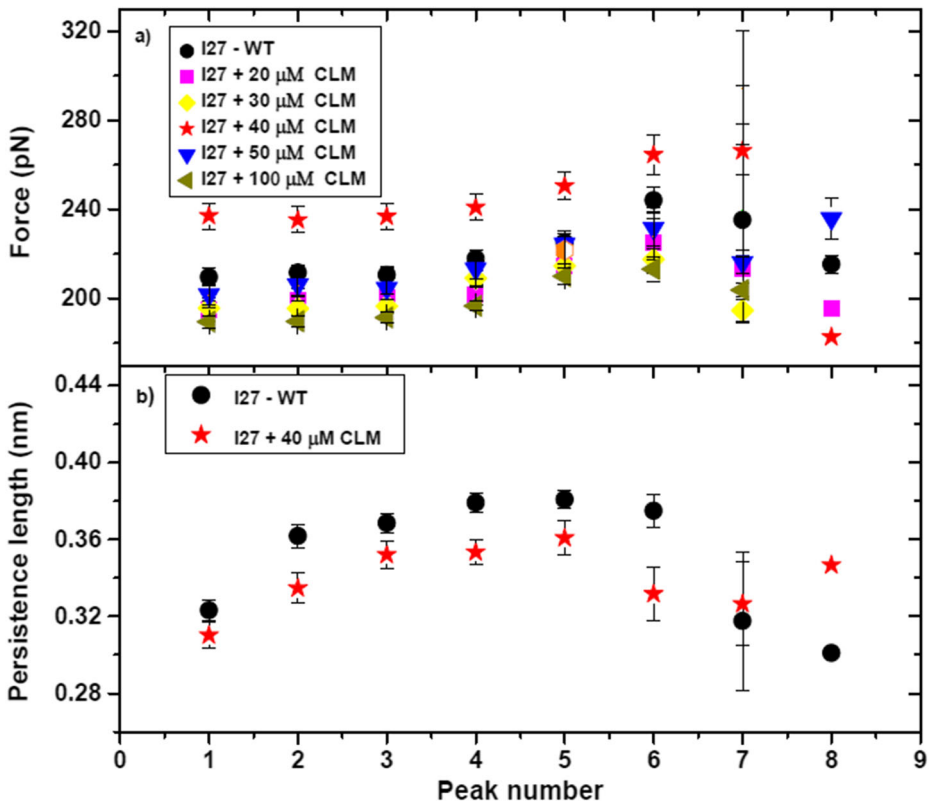


Fig. 5 Representative measurements for the unfolding of polyprotein without and with the drug **a** I27-WT, and **b** I27 with 40 μM CLM. The force required to unfold the domain is more for I27 with 40 μM CLM compared to I27-WT. Solid red lines represent the Worm-like chain model fitted to the force versus extension curve

Furthermore, we fitted a Worm-Like Chain (WLC) model (2) to the force-extension data of the unfolded domains of I27. This gives

$$F(x) = \frac{k_B T}{P} \left[\frac{1}{4(1 - x/L_c)^2} - \frac{1}{4} + \frac{x}{L_c} \right] \quad (2)$$

where F is the force, x the vertical tip position, k_B the Boltzmann constant, T the absolute temperature, P the persistence length, and L_c is the contour length of the polymer.

The persistence length, which characterizes the entropic elasticity of the unfolded chain, and the contour length, which is the measure of maximum physical possible extension of the chain, were used as fit parameters. Force curves with more than 6 events were considered for the analysis. We analyzed 160 force curves for I27-WT and 98 curves for I27 with 40 μM CLM concentration. Figure 6b shows the persistence length change on the addition of 40 μM CLM to the I27 protein. The graph shows that persistence length decreases with the addition of CLM for each unfolding domain. It suggests softening of the chain. Such softening could also be the result of enthalpic contribution to the chain elasticity due to binding of CLM molecule to the chain. This indicates that the CLM remains bound to the chain even after the domain is unfolded.

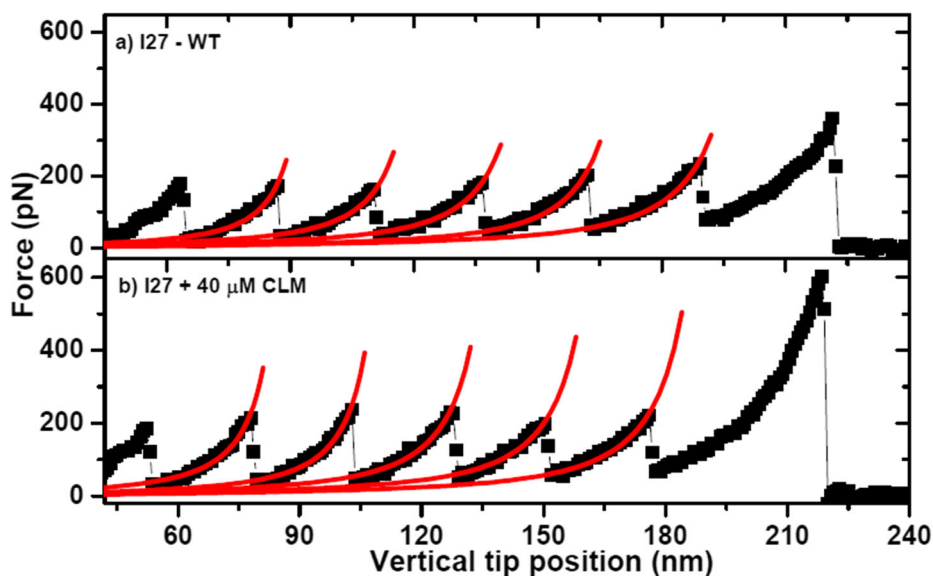


Fig. 6 The unfolding force for each domain and the persistence length of each domain. The peak number suggests the number of unfolded domains. The sample size is 160 and 98 for the I27-WT and I27 with 40 μM CLM respectively. **a** The domain-wise unfolding force after the addition of the drug at various concentrations. The unfolding force is higher for 40 μM CLM concentration. The difference is of ~ 25 pN for each domain; the error bars are standard errors, which are standard deviation divided by square root number of measurements N . **b** The domain-wise persistence length of I27-WT and I27 with 40 μM CLM. The error bars are standard errors

3.3 Equilibrium denaturation experiment

We performed equilibrium denaturation experiments at a fixed protein concentration (40 μM) with increasing GdmCl concentrations and monitored the denaturation of protein with varying CLM concentrations. The denaturation data obtained from fluorescence spectroscopy is plotted in terms of denatured fraction (f_D),

$$f_D = \frac{Y - Y_N}{Y_D - Y_N} \quad (3)$$

where Y is the observed fluorescence intensity at a wavelength where fluorescence intensity at maximum GdmCl concentration (6M)—fluorescence intensity at minimum GdmCl concentration (0M) is maximum. Y_N and Y_D are the fluorescence intensity values obtained for the native and denatured states respectively. The values of Y_N and Y_D were calculated by linear extrapolation in the transition region at low and high denaturant concentrations.

Figure 7a shows the denatured fraction at a particular denaturant concentration. The two curves represent unfolded fractions as a function of denaturant concentration. There is a shift in the GdmCl concentration when 40 μM CLM is added to I27 protein. This shift is towards the higher GdmCl concentration. For other concentrations below or above 40 μM , the curves do not shift by a large amount. This indicates that the chemical stability of the protein is enhanced at 40 μM drug concentration.

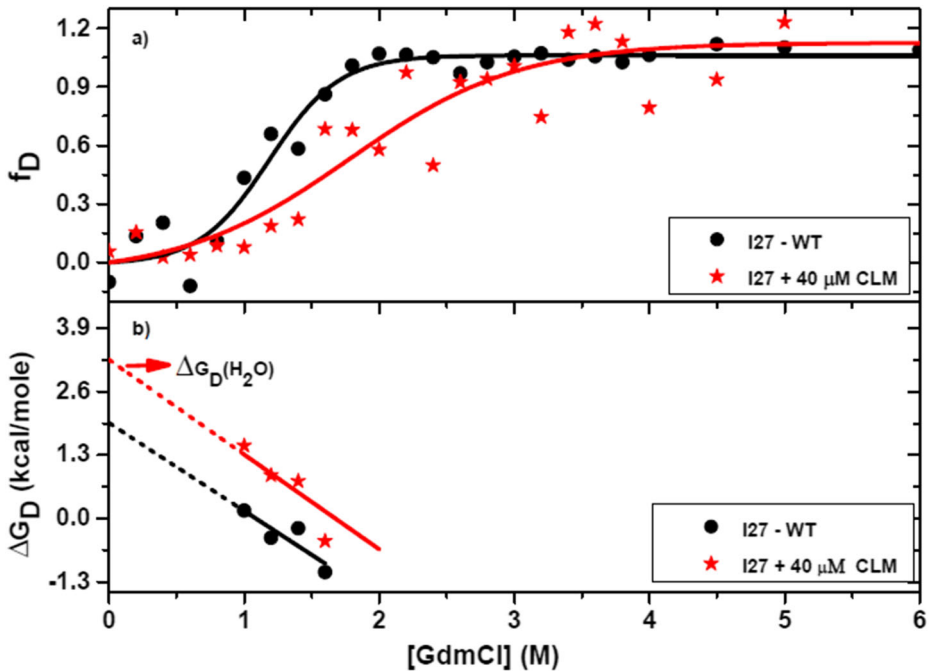


Fig. 7 Equilibrium denaturation experiments on monomers of I27-WT and I27 with different drug concentrations followed by Trp emission fluorescence spectroscopy. The denaturation data is plotted in terms of denatured fraction (see (3)). **a** The data in black filled squares are I27-WT and filled red circles are of I27 with 40 μM CLM concentration. Solid lines are fit to a two-state model. **b** Plot of ΔG_D versus GdmCl molarity for calculation of $\Delta G_D(H_2O)$ (see (6))

The equilibrium denaturation data was analyzed using methods described previously [32, 33]. The equilibrium constant K_D between native and denatured state is calculated using

$$K_D = \frac{f_D}{1 - f_D} \quad (4)$$

Values of f_D ranging from 0.4 to 0.8 were used for the K_D calculation. K_D values were then converted to ΔG_D . Free energy change, ΔG_D , is then calculated using (5).

$$\Delta G_D = -RT \ln K_D \quad (5)$$

The free energy of stabilization, i.e., $\Delta G_D(H_2O)$, was then calculated by linear extrapolation to zero denaturant concentration when a graph is plotted between ΔG_D and denaturant concentration

$$\Delta G_D = \Delta G_D(H_2O) + m(GdmCl) \quad (6)$$

where m is the slope of the line.

Figure 7b shows the free energy of stabilization for I27-WT and I27 with 40 μM CLM concentration.

The value of $\Delta G_D(H_2O)$ for I27-WT is found to be 1.95 ± 0.93 kcal/mole, while for I27 with 30, 40, and 50 μM is 1.79 ± 0.67 kcal/mole, 3.25 ± 0.63 kcal/mole, and 2.07 ± 0.62 kcal/mole, respectively. This indicates that at 40 μM drug concentration, the stability of the I27 domains is enhanced.

4 Discussion

Previously, protein-drug interactions have been investigated by various methods such as UV-visible absorption spectroscopy, fluorescence, circular dichroism (CD), isothermal calorimetry (ITC), and 3D fluorescence spectroscopy. Interaction of CLM with serum albumin proteins (both HSA and BSA (bovine serum albumin)), lysozyme, hemoglobin, and bovine pancreatic system was studied by these methods [25–27, 34–36]. We investigated the binding of CLM with titin in the current work. The fluorescence data and the Patch-Dock results suggest that quenching is due to binding of CLM near Trp. The mechanism of fluorescence quenching was also studied. The results show that quenching is due to the formation of the I27-CLM complex.

The mechanical properties of I27 in the presence of calcium were studied previously, which shows an increment of unfolding force by 40 pN and stiffness of unfolded domain is inferred through persistence length [20]. Another investigation on mechanical protein fibronectin as well as the membrane protein bacteriorhodopsin has shown that small osmolytes enhance stability of folded domains and reduce the persistence length of the unfolded chain [37–39]. It is argued that in the presence of such small molecules, the protein chain tends to form coils and it reduces the persistence length [40]. The mechanical stability of the protein is enhanced in such situations due to increase in chemical potential of denatured versus the folded state. The interaction of CLM with I27 shows a similar result. However, it is unclear if such effects are working in the case of CLM stabilizing the protein since we see this effect at a much lower concentration of CLM compared to these osmolytes. Our single-molecule measurements show that unfolding force is increased by 25 pN with 40 μ M drug thereby stabilizing the protein. Furthermore, stability is confirmed by equilibrium denaturation experiments. Denaturation experiments also show the stability at 40 μ M drug concentration.

One of the limitations of the AFM-based pulling experiments is the validity of WLC model to infer persistence length. WLC is successful in situations wherein the enthalpic contribution to the free energy is negligibly small. This is true in cases such as homopolymers in good solvents. The measurement of persistence length is thus likely to be riddled with errors if enthalpy is significant. However, the mechanical stability of individual domains is reported accurately by AFM pulling experiments. The stabilization of I27 with CLM in our study is also corroborated with ensemble studies of single monomers. We note here that in order to probe changes in mechanical properties with such drug molecules at low concentrations, it is important to provide evidence of binding of drug molecules with complementary methods such as fluorescence quenching.

Besides possessing antibacterial activities, CLM acts as a muscle relaxant [21]. Its effect is found to be direct on muscles and it is concentration-dependent. Fluorescence quenching experiments show that the drug binds to the I27 protein in the vicinity of Trp. AFM and equilibrium denaturation experiments suggest that binding results in both chemical and mechanical stabilization of the protein. Physiologically, it may affect the muscle function in a significant way. Our results are a step forward in terms of understanding the effect of CLM on the muscle sarcomere at the molecular level.

5 Conclusion

In this study, single-molecule and bulk measurements were performed to study the interaction of CLM with I27. The Stern-Volmer plot shows that protein-drug complex forms in

a certain range of drug concentration. The increase in unfolding force in single-molecule study also shows that binding takes place at a particular concentration (40 μM). However, there was no change in average unfolding force for other drug concentrations. Binding of protein with drug results in increasing the unfolding force of 25 pN. Chemical denaturation experiments show that the drug enhances chemical stability at this concentration. The higher unfolding force indicates that protein at 40 μM of drug concentration does not yield easily to external forces.

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Declarations

Ethics approval This study does not contain any animal model/experiment and hence does not require any ethical approval.

Conflict of interest The authors declare no competing interests.

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