The Pharma Innovation Journal 2018; 7(11): 05-11

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(11): 05-11

www.thepharmajournal.com
Received: 04-09-2018
Accepted: 06-10-2018

Kunzes Dolma
CSIR-Institute of Microbial Technology, Chandigarh India

Jyoti Nagar
CSIR-Institute of Microbial Technology, Chandigarh India

Deb Endra Kumar Sahoo
CSIR-Institute of Microbial Technology, Chandigarh India

Ashish
CSIR-Institute of Microbial Technology, Chandigarh India

Correspondence
Ashish
CSIR-Institute of Microbial Technology, Chandigarh India

Tracking urea induced unfolding-refolding of six-domain protein gelsolin by SAXS data analysis

Kunzes Dolma, Jyoti Nagar, Deb Endra Kumar Sahoo and Ashish

Abstract
Is hydrophobic collapse model (HCM) is applicable to multi-domain proteins resulting from gene duplication/triplication? To probe this, we analyzed small angle X-ray scattering (SAXS) datasets on six-domain protein, gelsolin as function of increasing and decreasing urea. Kratky and Porod exponent plots and radius of gyration (R_G) values indicated that with increase in urea, gelsolin transformed from globular to Gaussian-chain like to unfolded state. Upon reversal by dialysis at 4°C, profiles reversed back, except in presence of Ca^{2+} ions or low pH or at increased temperature. Overall, our results uphold that HCM is extendable to gelsolin if done under native conditions.

Keywords: Small angle x-ray scattering; gelsolin; folding-unfolding

Introduction
Based on available literature, the accepted notion of urea induced unfolding of protein is that increasing concentration of urea redistributes/alters the hydration shell of protein enough to weaken hydrophilic interactions at surface [1, 2]. Eventually, unpacking of surface and secondary structural content leads to exposure of the hydrophobic core to bulk solvent. Apparently, reduction of urea in the medium allows the hydrophobic and hydrophilic contents of protein to re-partition based on basic principles and eventually the ensemble of conformations trace their way back to its folded state. This systematic burial of central hydrophobic which initiates the refolding has been termed as hydrophobic collapse model (HCM) [3, 4]. Using multitude of biophysical techniques including *in silico* simulations, these events have been followed for different proteins which have been relatively well-characterized orthogonally [5, 6] Further, the proteins have been mostly single domain ones and techniques were mainly indirect read-out of the shape properties like distance between two or more fluorescence reporter groups and/or transient loss of α-helical or β-sheet content [7, 9]. We wondered whether HCM is extendable to multi-domain protein(s), particularly when the domains closely spaced, similar in content, structure and size. Probably, proximity of two hydrophobic cores may allow them to interact during collapsing events and one may end up with an alternatively folded protein? Though urea unfolding and refolding has been one of the basic and highly employed protein chemistry methodologies, literature did not show any report on encountering any grossly alternatively folded protein upon refolding. Probable reasons are: 1) single or two domain proteins selected for exploration may not have many equally probable junction points in their refolding pathway, and/or 2) biophysical parameters being read were localized effects, not a summation or average of overall or wide-range parameters beyond one or two sites of interest, and/or 3) hydrophobic collapse is very specific and scalable as per domain size being co-wired with Anfinsen's hypothesis [10].
In order to explore above query, we opted to follow urea induced unfolding and refolding by dialysis of gelsolin, a six-domain protein which has homologous domains by virtue of evolution by gene triplication followed by duplication[11]. All six domains have very similar content and structure, and are connected by linkers of variable lengths. In solution, it adopts a tight compact conformation in solution in absence of its shape-function activators. Earlier, reversible nature of complete opening of all six domains by Ca^{2+} ions [11] or low pH [12] or increased temperature has been established [13]. Furthermore, functional gelsolin has been recovered from inclusion bodies by employing urea induced denaturation followed by refolding [14]. The resumed functional status of refolded proteins shown in these publications affirm that this multi-domain protein can undergo unfolding-refolding steps. This further supported choice of this protein for these experiments. SAXS was considered as a preferred

technique in this work, since in same dataset it provides how different interatomic vectors present inside the scattering shape of molecules in solution, are related to each other. Essentially, each SAXS intensity profile as a function of momentum transfer vector provides an integrated global read-out of the scattering molecules without the need for selective or combination of reporter groups in the biomolecules. Mapping of Porod exponent [15] from SAXS datasets revealed that protein molecules transform from globular to Gaussian-chain like to unfolded shapes with increase in urea, and in some conditions, they folded back to functional shapes.

Materials and Methods

Gelsolin for Experiments

Following well-optimized protocols in our lab and as published before, His-tagged gelsolin was expressed, purified and characterized for all the SAXS experiments [16]. For urea denaturation experiments, gelsolin in Tris-Cl Buffer pH 8 was dialyzed against same buffer with increasing or decreasing amounts of urea. Four different concentrations of protein from 3 to 10 mg/ml was used to obtain shape properties at different molar ratios of urea to gelsolin. For forward dialysis (increasing amount of urea), three buffer changes of about 20 minutes were done. 10 kDa cut-off membrane was prepared for dialysis by boiling in 1 mM EDTA solution. For reverse dialysis (decreasing amount of urea), five buffer changes of about 20 minutes each were done. Final buffer for each dialysis was considered as matched buffer to subtract of contribution of constituents in buffer to SAXS data profiles.

SAXS data collection and data processing

All SAXS experiments were carried out on in-house instrument (SAX Space, Anton Paar) using line collimation optics. During scattering data collection, temperature was maintained at 10°C. About 250 µl of protein sample(s) and matched buffer(s) from different steps of dialysis were placed in 96-well format inside Waters Autosampler maintained at 10°C covered with thin aluminum foil. The autosampler was controlled by SAX Space Controller software, and its operations were pre-programmed and tested with control samples. In between loading of each sample, the autosampler was programmed to wash the delivery lines and capillary with 30% 2-propanol solution in water and dried using air. Post-loading of sample or buffer, SAXS data was collected for three exposures of 15 minutes and profiles were averaged. Smooth operation of programmed process or replenishment of wash buffers was ensured with intermittent manual observations. All processing were done in batch mode. SAXS treat program was used to correct for beam position for all datasets. SAXS quant program was employed to subtract the contribution from matched buffers, and desmear the I (Q) profiles.

SAXS data analysis

All data files were manually examined for lack of aggregation or any unusual profile using PRIMUSQT program[17] in ATSAS 2.8.1 suite of programs. Same program was also used for visual analysis of lack of aggregation and inherent disorder via Kratky plot. AUTORG program was used to estimate radius of gyration (R_G), usable intensity at lowest Q value (Q_{min}), and extrapolated intensity at zero angle of scattering (I_0) values in batch mode. SCATTER program[18] was used for calculated of the Porod exponent[15] following the step-wise instructions. For making figures in this work, Origin5 software was used.

Results and Discussion

SAXS Data Analysis for [Urea]/[Gelsolin] Samples

Samples of full-length gelsolin at concentrations of 3, 6, 8 and 10 mg/ml in Tris buffer pH 8 having 2 mM EGTA were dialyzed against same buffer but having 0, 0.25, 0.5, 1, 2, 3, 4, 5, 6 and 7 M of urea. These samples were expected to represent protein samples with progression towards unfolding. Samples at 7M urea were systematically dialyzed with buffers having lower urea concentrations to obtain insight into refolding. SAXS I(Q) profiles were collected on these samples to understand shape parameters and folded nature of the gelsolin molecules at different molar ratios of urea. The I(Q) profiles acquired for 10 mg/ml of gelsolin in buffers varying in urea amounts have been shown in Figure 1A. $Log_{10}I$ (Q) vs. $Log_{10}Q$ supported that there was no aggregation in samples during the denaturant range studied both during unfolding and refolding [12, 16, 19]. Kratky plots of the datasets revealed that during unfolding peak like profile of gelsolin progressively transformed into hyperbolic profile supporting conversion of globular scattering shape into inherently disordered one[20] (Figure 1B). For 10 mg/ml gelsolin sample, the hyperbolic profile of the $I(Q)*Q^2$ vs. Q clearly supported loss of globular shape with presence of 3M or more of urea in buffer. Interestingly, upon removal of urea from samples and induction of refolding, the inherently disordered protein changed back into globular shape. Qualitatively, it appeared that reduction of urea to same amounts (3M or less) induced adoption of globular shape by gelsolin. This provided first insight into forward and backward transformation of a multi-domain protein, gelsolin from globular to disordered shape and back as a function of urea in buffer.

To get a semi-quantitative estimation of the extent of change from globular to disordered shapes, Porod exponent of the datasets were estimated (Figure 1C). Results showed that for 10 mg/ml gelsolin samples, the data points plateaued for exponents of 3.7 to 2 with increase in urea concentration from 0 to 7 M. For example, the Porod exponents for 0, 2, 4 and 7 M datasets were 3.7, 2.7, 2.1 and 2, respectively (Figure 1C). In correlation with Kratky plots, deviation or decrement from Porod exponent of 4 indicated increasing deviation from globular profile. Comparing with Kratky plot, peak like profile or indicative of globular shape was comparable with Porod exponent value of 4 to 3, and hyperbolic curves indicative of inherently disordered or Gaussian chain like to unfolded shape were accompanied with decrement of Porod exponent from 3 to 2. It has been earlier explained that decreasing values of Porod exponent physically represent stages when it is difficult to identify a clear contrast between the edge of the scattering particle and the bulk buffer [15, 21]. Having seen that SAXS data analysis based tracking of refolding steps of gelsolin from its urea unfolded state essentially provides indistinguishable results from forward unfolding states, we conclude that estimated Porod exponent is a reliable way to represent the information shown from Kratky plots. It is pertinent to mention here that interpretation of variation in increasing Q values in exponent plots depends on data quality at mid and wide angles and thus it is more reliable in samples with higher concentrations. In our case, data did turn noisy at higher urea concentrations as can be seen for Porod plot for sample at 7 M, but our interpretations are semi-quantitative and relative in nature.

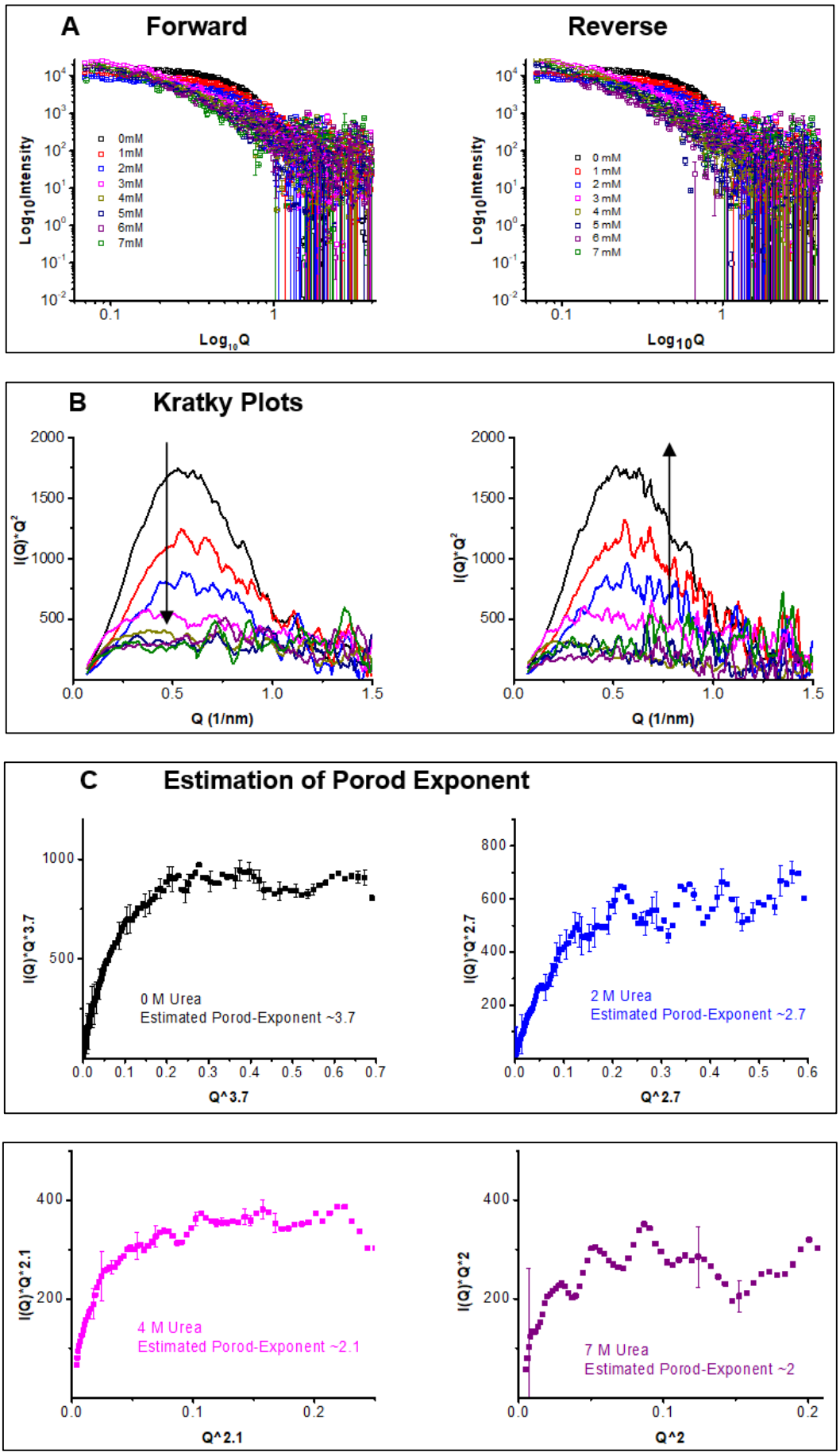


Fig 1: (A) SAXS intensity profiles of gelsolin at 10 mg/ml in buffers having varying concentration of urea are presented. Forward and reverse sets represent increasing and decreasing amounts of urea. (B) Kratky plots of the SAXS datasets shown in above panels are plotted. The arrows show the direction of the decrement and re-gain of peak height. (C) Porod plots for few datasets are shown with the Porod exponent estimated using SCATTER program

Porod exponent and average R_G values of different gelsolins

To have some parity between experiments and different sizes of protein being studied, we converted the different molar ratios of urea to gelsolin to number of urea molecules to that of residues in different gelsolins. Mapping of changes in the estimated Porod exponent as a function of number of urea molecules per residue of gelsolin indicated that the protein is initially globular and with increase in urea follows a two state transformation to unfolded shape with mid-point of transition around 10-20 urea molecules per gelsolin (Figure 2 Top Left). Interestingly, reversal of the trend indicated that the globular or folded state is achieved slightly faster than unfolding. Using the same datasets, variation of R_G was estimated which indicated a three-state opening or unfolding, and a two-state refolding process. For gelsolin, three-state opening was seen earlier for Ca^{2+}-binding induced shape changes[11], and presence of middle stage indicated that some shape changes occur between first stage of unfolding and then later steps. Any case, the mid-point of changes in the average R_G of particles in ensemble at different urea concentration appeared close to 10-20 urea molecules per residue of gelsolin, in-sync with above plot for change in inherent disorder.

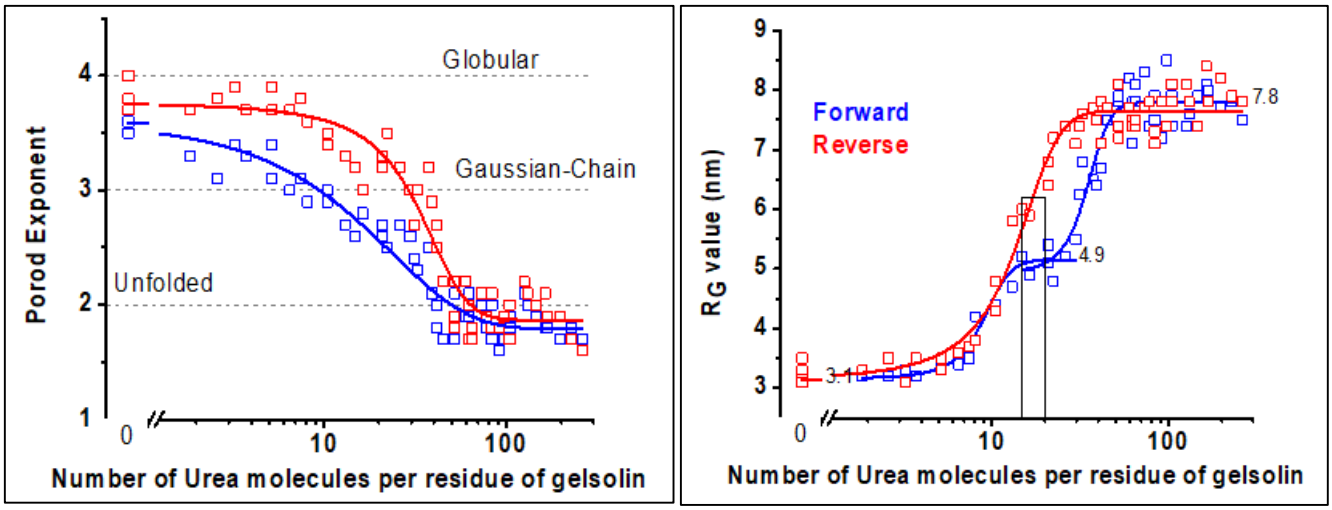
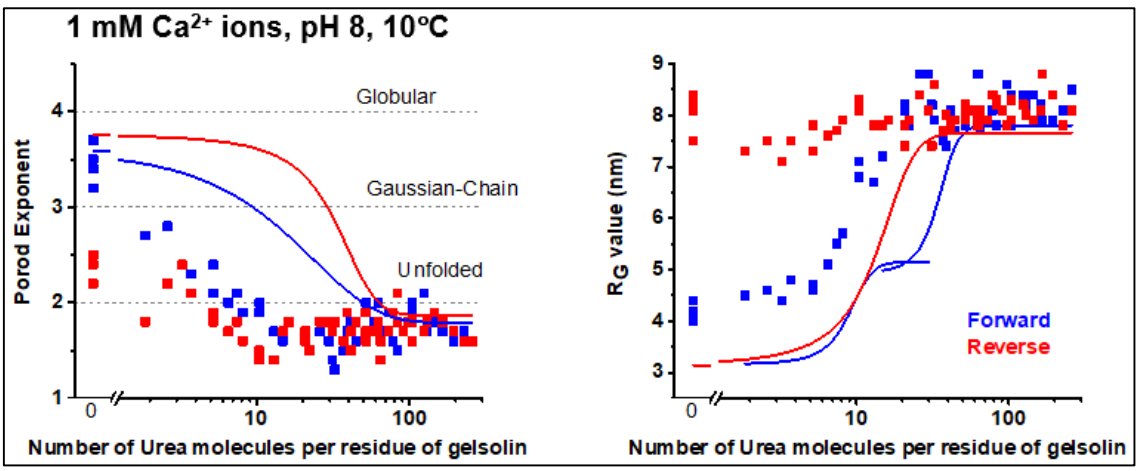


Fig 2: Variation in the estimated Porod exponent values (above) and R_G values (below) as a function of number of urea molecules per residue of gelsolins have been plotted here. Sigmoidal fits were done to understand the trend in increase and decrease. The open black rectangles indicate the approximate mid-points of transitions

Effect of Ca^{2+} ions or Low pH or Increased Temperature

Earlier, it has been established that Ca^{2+} ions, low pH and temperature can induce shape changes in gelsolin(s) which makes them competent to depolymerize F-actin, and these changes were found to be reversible. In this work, having seen that urea can unfold and proteins can refold, we wanted to explore how these activators of gelsolin will affect the urea induced folding and refolding behaviour. We prepared samples with 1 mM free Ca^{2+} ions and pH 6 with no Ca^{2+} ions. Another set of samples were dialyzed with (increasing and decreasing amounts of) urea having no free Ca^{2+} ions and pH 8, but at 35°C. For different samples, SAXS datasets were acquired as before and analyzed for their Porod exponent (and R_G values) (Figure 3). Data analysis brought out that in presence of 1 mM free Ca^{2+} ions or pH 6 or at 35°C, all three gelsolins showed a faster rate of unfolding and inability to refold correctly compared to their profiles in absence of these activators. R_G values mapped for gelsolin were found to be in agreement with estimated Porod exponent values that the disordered or unfolded states were achieved faster by Ca^{2+} or pH or temperature activated gelsolin, and refolding could not happen upon dialyzing out the urea. These results concluded that hydrophilic opening or hydrophobic collapse processes are tampered if factors which induce re-organization of shape of gelsolin.



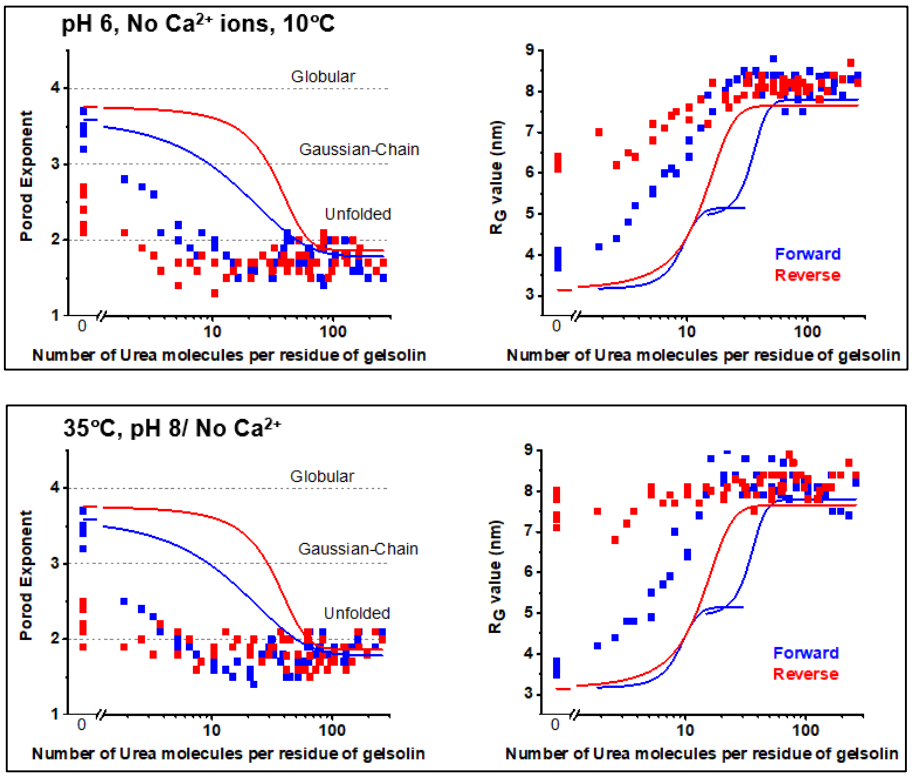
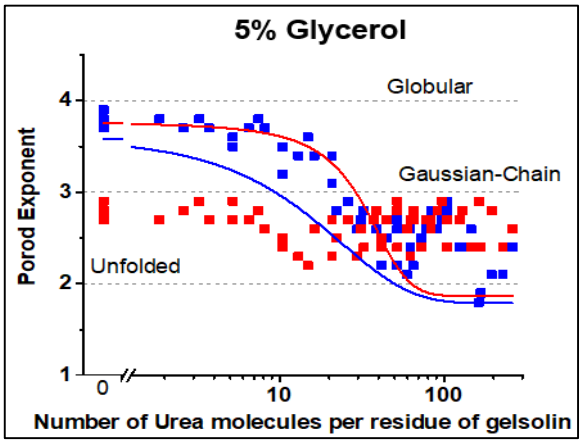
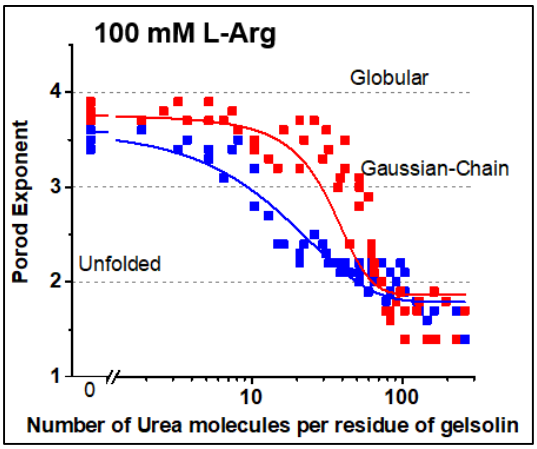


Fig 3: Top to bottom are panels of changes in estimated Porod exponent values for gelsolin and R_G values for gelsolin as a function of urea in buffer plus the mentioned activator have been plotted here. Solid blue and red squares represent the estimated value for a ratio of urea per residue in protein towards increasing and decreasing urea, respectively. Solid lines represent the profiles for proteins in absence of these activators (blue for unfolding and red for refolding)

Effect of Osmolytes which Stabilize Hydration Shell

With a thought whether the deviations in unfolding-refolding process of gelsolin occurred due to conformational rearranging molecules/conditions or presence of any additional osmolyte will vary the process, we acquired SAXS datasets from gelsolin in varying concentration of urea and added low amounts of some common protein stabilizers – 5% glycerol, 100 mM L-Arg and 4% sucrose (Figure 4). Porod exponent analysis brought out that glycerol slowed down both the process of unfolding as well as refolding for full-length gelsolin. Data analysis of samples containing L-Arg indicated that it did not affect globular to unfolded shape transformation and reversal for gelsolin. Samples supplemented with 4% sucrose brought out interesting trend for gelsolin that sucrose significantly slowed unfolding for gelsolin, but the refolding steps were very close to unfolding steps. This indicated that osmolytes known stabilize the hydration shell of the protein from bulk buffer can slow down the process of unfolding-refolding, making the events more specific.





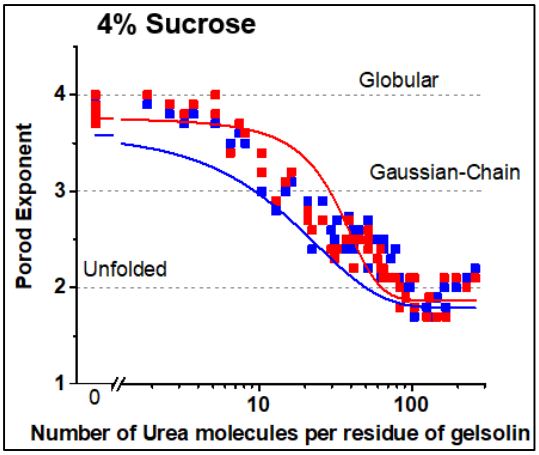


Fig 4: Top to bottom are panels showing trends in changes in estimated Porod exponent values for gelsolin as a function of urea in buffer plus mentioned osmolytes have been plotted here. Symbols and lines are as mentioned for Figure 3

Conclusion

This work based on extensive data collection and analysis was done to probe if denaturant induced unfolding and refolding is possible for multi-domain proteins with very domains separated variable sized/constitution linkers. A protein which has evolved by gene triplication followed by duplication named gelsolin was used to track its urea induced unfolding and folding. SAXS was employed as a tool as it is capable of mapping average shape parameters of ensemble of conformations in solution without need for any labelling or reporter groups. Porod exponent – a measure of unfolded state of shape was estimated which provided a unique way to monitor transformation of globular shapes into Gaussian-chain like disordered shape to unfolded shapes, and backwards. One of the intriguing results was that about 20 urea molecules are required per residue of protein to unfold half the population. This observation supported that with increase in urea, first probably the domains dissociate and then they unfold. Refolding steps probably initiate with formation of local folds leading to domains and then collapse of domains to form globular profile. This further reinforced Anfinsen's theory that refolding is coded in the primary structure of protein. Though not described here, F-actin depolymerization assays confirmed that the refolded gelsolin which achieved Porod exponent similar to untreated gelsolin displayed activity similar to untreated gelsolin (post addition of 1mM free Ca^{2+}). Though expected from earlier publications on refolding gelsolin from inclusion bodies, our results reinforced the fact that despite similarity in sequence content, the six domains of gelsolin did not "inter-fold" when refolding. Porod exponent, R_G values and F-actin depolymerizing data confirmed that addition of shape reorganizing elements specific to gelsolins alter the path between unfolding-refolding, yet osmolytes which stabilize hydration layer around proteins allowed native-like partitioning of hydrophilic and hydrophobic residues. As known, the relative positioning of domains in space are significantly altered by Ca^{2+} ions or low pH or temperature, very likely these conformational rearrangements coupled with urea induced progressive changes in structure substantially alter the information coded in primary structure. Thus, they interfere and induce deviation in the unfolding-refolding pathways. Finally, ability of sucrose to induce tracking of similar events during urea induced unfolding and refolding connects with the fact that injectable gelsolin, Solinex^{TM} is best formulated in about 40% sucrose during lyophilization and storage. Overall, we set a precedence for more work in this direction possibly using other protein systems, but we provide first insight on how unfolding can be tracked using SAXS, and in verifying how hydrophobic collapse model works and its limitations.

Author contributions

A conceived the idea and designed experiments. KD and JN did the experiments. A repeated some experiments, processed and analyzed SAXS data to write the manuscript. DKS provided guidance in fermentation based over-expression of gelsolin(s) used for experiments.

Acknowledgements

This work was primarily supported by CSIR INDIA via 12FYP UNSEEN (BSC0113) and OLP107 to A. KD and JN were supported by fellowships from DBT and UGC INDIA. This is IMTECH communication number: 064/2018

References

1. Canchi DR, Garcia AE. Cosolvent effects on protein stability. Annual review of physical chemistry. 2013; 64:273-293.
2. England JL, Haran G. Role of solvation effects in protein denaturation: From thermodynamics to single molecules and back. Annual review of physical chemistry. 2011; 62:257-277.
3. Arai M. Unified understanding of folding and binding mechanisms of globular and intrinsically disordered proteins. Biophysical reviews, 2018.
4. Udgaonkar JB. Polypeptide chain collapse and protein folding. Archives of biochemistry and biophysics. 2013; 531:24-33.
5. Bennion BJ, Daggett V. The molecular basis for the chemical denaturation of proteins by urea. Proceedings of the National Academy of Sciences. 2003; 100:5142-5147.
6. Kathir KM, Kumar TKS, Rajalingam D, Yu C. Time-dependent changes in the denatured state (s) influence the folding mechanism of an all β-sheet protein. Journal of Biological Chemistry. 2005; 280:29682-29688.
7. Horovitz A, Fersht AR. Co-operative interactions during protein folding. Journal of molecular biology. 1992; 224:733-740.
8. Wong KB, Clarke J, Bond CJ, Neira JL, Freund SM, Fersht AR, Daggett V. Towards a complete description of the structural and dynamic properties of the denatured state of barnase and the role of residual structure in folding. Journal of molecular biology. 2000; 296:1257-1282.
9. Mills BM, Chong LT. Molecular simulations of mutually exclusive folding in a two-domain protein switch. Biophysical journal. 2011; 100:756-764.
10. Anfinsen CB. Principles that govern the folding of protein chains. Science. 1973; 181:223-230.
11. Ashish Paine MS, Perryman PB, Yang L, Yin HL, Krueger JK. Global structure changes associated with ca2+ activation of full-length human plasma gelsolin. The Journal of biological chemistry. 2007; 282:25884-25892.
12. Garg R, Peddada N, Sagar A, Nihalani D. Ashish Visual insight into how low ph alone can induce actin-severing ability in gelsolin under calcium-free conditions. The Journal of biological chemistry. 2011; 286:20387-20397.
13. Badmalia MD, Singh S, Garg R, Ashish Visualizing temperature mediated activation of gelsolin and its deactivation by pip2: A SAXS based study. Scientific reports. 2017; 7:4670.
14. Yu FX, Zhou DM, Yin HL. Chimeric and truncated gcap39 elucidate the requirements for actin filament severing and end capping by the gelsolin family of proteins. The Journal of biological chemistry. 1991; 266:19269-19275.
15. Rambo RP, Tainer JA. Characterizing flexible and intrinsically unstructured biological macromolecules by sas using the porod-debye law. Biopolymers. 2011; 95:559-571.
16. Peddada N, Sagar A, Rathore YS, Choudhary V, Pattnaik UB, Khatri N, Garg R, Ashish Global shapes of f-actin depolymerization-competent minimal gelsolins: Insight into the role of g2-g3 linker in ph/ca2+ insensitivity of the first half. The Journal of biological chemistry. 2013; 288:28266-28282.

17. Konarev PV, Volkov VV, Sokolova AV, Koch MHJ, Svergun DI. Primus: A windows pc-based system for small-angle scattering data analysis. J Appl Crystallogr. 2003; 36:1277-1282.
18. Forster S, Apostol L, Bras W. Scatter: Software for the analysis of nano- and mesoscale small-angle scattering. J Appl Crystallogr. 2010; 43:639-646.
19. Jacques DA, Trewhella J. Small-angle scattering for structural biology--expanding the frontier while avoiding the pitfalls. Protein science: a publication of the Protein Society. 2010; 19:642-657.
20. Pandey K, Dhoke RR, Rathore YS, Nath SK, Verma N, Bawa S, Ashish Low ph overrides the need of calcium ions for the shape-function relationship of calmodulin: Resolving prevailing debates. The journal of physical chemistry B. 2014; 118:5059-5074.
21. Grant TD, Luft JR, Wolfley JR, Snell ME, Tsuruta H, Corretore S, *et al*. The structure of yeast glutaminyl-trna synthetase and modeling of its interaction with trna. Journal of molecular biology. 2013; 425:2480-2493.