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Unusual Domain Movement in a Multidomain Protein in Presence of Macromolecular Crowders

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Abstract

Domain movements play a fundamental and critical role with regards to the specific biological function that multidomain proteins have evolved to perform. Significant amount of research has been carried out to investigate the effects of macromolecular crowding agents, mostly on single domain proteins, thereby furthering our appreciation for the crowding phenomenon. However similar studies on proteins having multiple domains are relatively scarce. Using the plasma protein human serum albumin (HSA), as the protein of interest, we have probed the influence of Dextran based crowding agents (Dextran 6, Dextran 40, Dextran 70) on the relative movements of domains I and II using FRET, with Trp-214 in domain II acting as the donor and acrylodan (Ac) covalently attached to Cys-34 of domain I, as the acceptor. Amongst the higher molecular weight crowders, while both Dextran 70 and Dextran 40 induced significant decrease in the distance between the aforesaid domains, however for the latter macromolecular crowder (Dextran 40), beyond 50 g/L, no change in domain separation was observed even upto concentrations of 175 g/L. On the other hand, contrary to our expectations, Dextran 6, having the highest packing density by virtue of it being the smallest crowding agent used, provided an asymmetric excluded volume which resulted in forced elongation of HSA along the Trp-Ac FRET axis. Additionally both chemical and thermal studies performed at varying concentrations of the chemical denaturant urea, reveal unusual movements of the two domains, an aspect that can have important implications with regards to HSA being an avid transporter of fatty acids, with the binding of latter being known to invoke appreciable domain displacements. We hypothesise that we see a distinct crossover from entropy dominated depletion effects in case of Dextran 6 to significant enthalpic contribution for Dextran 70 with Dextran 40 lying midway between these two crowders, having characteristics of both.

Introduction

Majority of the proteins must fold from a relatively extended unfolded state to a unique compact native structure to exert their biological activity. Extensive research has been carried out to investigate the varied underlying mechanisms and driving forces that are important for proteins, having different degrees of structural complexity, to fold in a physiologically relevant timescale. While most of these studies have been performed in dilute buffer media, the intracellular environment is however significantly different in being highly crowded due to the presence of large amounts of macromolecules[1] (proteins, nucleic acids, ribosomes and carbohydrates) that occupy ~10 - 40 % of the total cellular volume. These macromolecules, popularly termed as crowding agents, through their excluded volume effect, reduce the space available to the biological molecule of interest. Such a phenomenon has been shown to affect a plethora of biologically relevant processes, with the protein folding-unfolding phenomenon being a prime example, since the latter is almost always accompanied by a measurable volume change. Thus it follows that since the denatured population of proteins in general occupy larger conformational spaces as compared to native structural ensemble, hence these are likely to be more influenced by the excluded volume phenomenon [2-5]. Indeed, protein stability studies have revealed that macromolecular crowding agents in general delay the onset of denaturation by entropically destabilizing the unfolded ensemble because of lack of conformational space for the extended structures [6-8].

Irrespective of whether one uses dilute buffer solutions or the crowded media, most of the folding studies have focussed on either single domain proteins[9] or those that are a part of bigger proteins having multiple domains[10- 11] or involving DNA[12]. Understanding the complex landscape of a multidomain protein, wherein the synergy in folding of the different domains and

the underlying interdomain interactions play a major role, in itself presents a huge challenge, as can be aptly figured out from the paucity of such studies in literature[13]. Notwithstanding the magnitude of difficulty involved, it should be realized that proteins having multiple domains make up a significant fraction of the full repertoire of proteins found in nature; thus deciphering their internal dynamics such as relative movements of domains, that can be very critical for proper functioning of the protein, especially in physiological environments that are perennially crowded, becomes an immediate necessity. With this goal in mind, we have, in this paper, investigated the unfolding of the multidomain (three domains) protein human serum albumin (HSA) and the relative movements of two of its domains (domains I and II), in presence of Dextran based macromolecular crowders having different average molecular weights (Dextran 6, Dextran 40 and Dextran 70). Human serum albumin (HSA) is a monomeric protein of 585 amino acid residues and is composed of three homologous α-helical domains I–III (Figure 1), which is further divided into sub domains A and B[14-15]. The most desirable property of human serum albumin is its ability to bind to a variety of endogenous and exogenous ligands and fatty acids, and much of the interest in this protein stems from its effects on drug delivery[16-19].

The evolution/changes in the conformation of the protein in presence of the crowding agents were monitored using primarily using the intrinsic Trp fluorescence (HSA has only one tryptophan residue, Trp-214). In order to probe interdomain movements we have monitored FRET between the intrinsic fluorescence of tryptophan (Trp-214) residue of HSA and an external probe Acrylodan, the latter being covalently linked to the free cysteine (Cys-34) of domain I of the protein, over a range of crowder concentrations and also subjecting HSA to both chemical and thermal denaturation. Our results show interesting crowder dependent profiles in the manner in which the distance between the Trp-Ac FRET pair gets modulated. Taken together

these reveal possible mechanistic aspects by which these crowding agents of different average molecular weights influence the domain separation. We hypothesise that we see a distinct crossover from entropy dominated depletion effects in case of Dextran 6 to significant enthalpic contribution for Dextran 70. Such conformational modulations can be of great physiological significance with regards to fatty acid or drug binding to HSA[18], that is, the function of HSA as a small molecule transporter.

1.1 Experimental Procedure

Chemicals

Essentially fatty-acid-free HSA, Urea, Dextran (6, 40, 70 kDa) were purchased from Sigma Aldrich chemicals Pvt. Ltd. (USA) and were used as received without purification. Sodium phosphate dibasic anhydrous (Na_2HPO_4) and sodium phosphate monobasic anhydrous (NaH_2PO_4) were purchased from Merck Specialities Pvt, Ltd. (Mumbai) and used as received. Acrylodan was purchased from Molecular Probes Inc. (Invitrogen, USA).

1.2 Protein labelling and purification

A stock solution containing 50 μM HSA was prepared in 50 mM phosphate buffer (pH = 7.0). A 50 μL aliquot of this solution was taken, and acrylodan (dissolved in the minimum volume of CH_3CN) was added such that the molar ratio of HSA to acrylodan was 1:1.28[20]. This mixture was stirred gently and maintained at 4°C for 12 h in dark. The labelled protein was then eluted through a G-25 sephadex column (molecular mass exclusion limit of 5000 for proteins) to separate it from any excess acrylodan molecules (unreacted). The concentration of the labeled

protein was found by using ε_{278} = 8,700 M^{-1} cm^{-1} and ε_{365} = 12,800 M^{-1} cm^{-1} for acrylodan and ε_{277} = 36,000 M^{-1} cm^{-1} for HSA[21]. The labelling efficiency was determined to be greater than 80%.

1.3 Sample preparation

The phosphate buffer solution was prepared by mixing definite weighed amounts of monobasic and dibasic phosphate salts in Millipore water. The pH of the resultant buffer was measured by a pH meter (pH 510, Cyberscan, Eutech Instrument). Concentration of urea was ascertained by measuring the refractive index of the urea stock solution using a refractometer (Krüss Optronics, Germany). Desired amount of crowding agents were weighed using an analytical balance (Precisa, Sweden) and dissolved in phosphate buffer solution. The resultant solution was kept at 100 g/L with respect to the crowder concentration throughout.

1.4 Spectroscopic Measurements

Absorption

Absorption measurements were performed in double-beam Shimadzu UV-VIS Spectrometer (UV-2450, Japan) using 1cm path length cuvettes. Absorbance values of the protein solutions were measured in the range of 200-600 nm and molar extinction coefficient for the HSA used was ε_{280} = 36500 $M^{-1}cm^{-1}$ [22].

Steady state Fluorescence measurements

Steady state fluorescence measurements were carried out on a Fluoromax-4 spectrofluorometer, (HORIBA Jobin Yvon, USA). The fluorescence spectra of protein samples at varying

concentrations of denaturant in presence and absence of crowders were measured using fluorescence quartz cuvettes. Prior to each experiment, the concentration of every sample was measured using the UV spectrophotometer. The fluorescence spectra of the protein samples were recorded at 25 °C with the temperature being maintained by a Peltier based controller and the protein concentration was maintained at 2 μM for all the experiments. The samples were allowed an equilibration time of 2 hours before acquiring their respective spectra. Samples containing unlabeled HSA were excited at 295 nm, and emission was collected from 305 nm to 450 nm in 1-nm increments with an integration time of 0.4 s, using a band pass of 4 nm in both the excitation and emission arms of the instrument. Acrylodan-labeled HSA was excited at 365 nm, with emission collected from 380 nm to 600 nm to monitor the acrylodan emission alone. For energy transfer experiments, the labeled protein was excited at 295 nm, and emission was collected from 305 nm to 550 nm.

Circular Dichroism

Far-UV CD experiments of HSA were carried out using an AVIV circular dichroism spectrometer (Model 420SF, Lakewood, NJ, USA). The samples were contained in quartz cuvettes of 1 mm path length. Thermal denaturation of HSA in the absence and presence of urea was monitored at 222 nm at a scan rate of 4 °C/min. Temperature was varied from 10 to 90 °C for all the thermal experiments.

Förster resonance energy transfer (FRET) analysis

Trp and acrylodan moieties form an efficient FRET pair (Supplementary Information Figure 1) and hence allows one to calculate the distance between the two domains of the protein during the course of chemically induced unfolding (with Trp being in domain II and acrylodan in domain I).The efficiency of the energy transfer (E) as a function of distance (r) between two probes can be expressed as follows

$$E = 1 - \frac{I_{DA}}{I_D} = \frac{R_0^6}{R_0^6 + r^6} \quad (1)$$

where R_0 (Förster radius) represents the distance at which energy transfer is 50% efficient, I_{DA} is the intensity of the donor in presence of the acceptor, and I_D is the unquenched donor intensity (that is in absence of the acceptor). The value of R_0 (Å) can be obtained from the equation below

$$R_0 = 9.78 \times 10^3 \left[J(\lambda) n^{-4} \kappa^2 \Phi_D \right]^{1/6} \quad (2)$$

Where $J(\lambda)$ is the overlap integral, n is the refractive index of the medium and κ^2 is the orientation factor between the donor and acceptor electronic transition dipole moments. For our study we have calculated the energy transfer efficiency from area under the curve of the labelled and unlabelled proteins.

The value of R_0 for native protein was first calculated in phosphate buffer and was determined to be ~ 25 Å using donor quantum yield of 0.14, a $J(\lambda)$ value of 1.18×10^{14} nm^4 M^{-1} cm^{-1}, and with κ^2 set to 2/3 and n set to 1.34. For different crowder solution in presence of increasing urea concentration R_0 and $J(\lambda)$ were also calculated using MATLAB R2013B software.

Thermodynamic analysis of chemical denaturation

The denaturation data obtained were analysed assuming one two-state equilibrium, according to the method of Pace et al. (1989), modified by Santoro and Bolen (1988)[23]. According to this method, the standard free energy of unfolding, $\Delta G^{\circ}_{F\to U}$ is considered to be a linear function of the concentration of the denaturant. The equilibrium constant, $K_{F\to U}$ evaluated in the transition region is converted to free energy data, $\Delta G_{F\to U}$ in this method and then these data was plotted as a function of denaturant concentration, *[D]* and extrapolating data to zero denaturant concentration. Mathematically

$$\Delta G_{F\to U} = \Delta G^{o}_{F\to U} + m_G[D] \qquad (1)$$

where $\Delta G^{\circ}_{F\to U}$, the intercept, is the free energy change for unfolding in absence of any denaturant, m_G, the slope is measure of the surface exposed of the protein during unfolding and $\Delta G_{F\to U} = -RT \ln K_{F\to U}$.

The spectroscopic signals of folding are given by the expression

$$F = F_{Folded} X_F + F_{Unfolded} X_U \qquad (2)$$

Where F is the measured spectroscopic intensity, F_{Folded} and $F_{Unfolded}$ are the spectroscopic intensities for the native and denatured states, and X_N and X_D stand for the mole fractions of these states, respectively. Both F_{Folded} and $F_{Unfolded}$ were assumed to depend linearly upon the concentration of the denaturant *[D]*:

$$F_{Native} = F_N + m_N[D], F_{Unfolded} = F_U + m_U[D] \quad (3)$$

Mole fractions (X_F, X_U) are related to the denaturation equilibrium constant by

$$X_F = 1/(1+K_{F\to U}) \text{ And } X_U = K_{F-U}/(1+K_{F\to U}) \quad (4)$$

From equation (1), (2), (3) and (4)

$$F_D = \frac{(F_N + m_N[D]) + (F_U + m_U[D])\exp\{-\Delta G^{o}_{F\to U}/RT - m_G[D]/RT\}}{1+\exp\{-\Delta G^{o}_{F\to U}/RT - m_G[D]/RT\}} \quad (5)$$

Where F_D is the value of the fluorescence intensity (area under the curve) at a given concentration of denaturant, *[D], R* is the gas constant, and *T* is the temperature in absolute scale. The remaining six terms are fitting parameters, where F_N and F_U are the values of the intensity

(area) extrapolated to zero denaturant concentration for the native and unfolded states, respectively; m_N and m_U are the slopes for the dependencies of F_N and F_U on denaturant concentration; $\Delta G^{\circ}_{F\rightarrow U}$ is the free energy of unfolding in absence of the denaturant; and m_G is the slope describing the dependence of $\Delta G_{F\rightarrow U}$ on denaturant concentration. The transition midpoint ($D_{1/2}$) values can be calculated by dividing $\Delta G^{\circ}_{F\rightarrow U}$ by $-m_G$. The variation of interdomain distance of HSA during the course of chemical denaturation in buffer and different crowded media was also fitted according to the equation (5). The free energy values obtained by fitting the unfolding profile and interdomain distances have been renamed as $\Delta G_{\text{global unfolding}}$ (kJ mol^{-1}) and $\Delta G_{\text{domain separation}}$ (kJ mol $^{-1}$) respectively.

Results and Discussion

The interdomain separation between domains I and II of HSA was investigated as a function of the chemical denaturant urea, both in presence and absence of crowding agents. Dextran 70, 40 and 6 were chosen as the macromolecular crowders based on their neutral and inert nature and thereby influencing the behaviour of proteins primarily via the excluded volume effect. The change in the emission intensity was monitored for tryptophan (Trp) in both the HSA and Ac-HSA. The Trp residue in the native, unlabelled protein had its emission maximum around 341 nm for buffer (in good agreement with earlier studies[24]) and the same was retained in presence of the crowders. To provide a detailed picture of the effect of crowders on the FRET modulation and hence the domain separation during the course of unfolding a wide range of crowder concentrations varying from 25 g/L to 175 g/L was used (enhanced scattering precluded studies at higher crowder concentrations).

FRET modulation during chemical unfolding

Labelling of the free cysteine moiety (Cys-34) of HSA with acrylodan (as the acceptor of Trp-214 fluorescence) has been widely used in the past to probe conformational changes and associated modulation in site-specific hydration dynamics of the serum albumin[21,25-26]. The emission spectrum of tryptophan and the absorption spectrum of Acrylodan (Ac) show appreciable overlap (Supplementary Information Figure 1) which is further supported by the high efficiency (E) of energy transfer (~0.65) observed in buffer for native HSA corresponding to a 25.5 Å separation between the FRET pair. With increase in urea concentration, the efficiency shows a steady decrease (from 0.65 at 0 M urea to 0.29 at 8 M urea) signifying a gradual movement of the FRET pair and hence the two domains (I and II) apart from one another (Figure 2A). Maximum change in interdomain distance (*r*) occurred between the urea concentrations of 2 M to 7 M (27.4 Å to 31.3 Å). There was a sharp increase in distance between domain I and II beyond 4 M that continued up to 6 M of urea and beyond which it remained almost constant (Figure 2A). Incorporation of the macromolecular crowder, Dextran 40 brings a dramatic decrease in interdomain separation even at a very low concentration. At 25 g/L Dextran 40 the interdomain distance for the native state of HSA decreases to ~ 23 Å and efficiency of energy transfer (E) increases about 15% than that of buffer (Figures 2B and 3 and Supplementary Information Table 1). With increasing denaturant concentration the domain separation increases and follows a trend quite similar to that of buffer. As the Dextran 40 concentration increases to 50 g/L there was a sudden drop in *r* (~ 23 Å to ~ 21 Å) corresponding to an increase in E (~ 0.75 to ~ 0.84) (Figures 2B and 3 and Supplementary Information Table 2). Beyond this concentration of Dextran 40 the efficiency of energy transfer between domains I and II remains almost constant (Supplementary Information Tables 3-7). From Figure 2B it is evident that for all the crowder (Dextran 40) concentrations, the distance increases as a function of urea and the manner in which

the FRET pair distance varied followed a sigmoidal pattern akin to global unfolding of proteins. This observation is not only indicative of the fact that movement of domain I with respect to domain II is cooperative in nature but also that this aspect of the unfolding process is preserved throughout for all the concentrations of Dextran 40 used here, that is, upto 175 g/L.

Increasing concentration of Dextran 70 was used to mimic the effect of higher molecular weight crowder on the interdomain separation during the urea induced unfolding of the serum protein. For native HSA the distance between domains I and II decreases from ~23 Å to ~ 18 Å as the Dextran 70 concentration increases from 25 g/L to 175 g/L (Figures 2C and 3). This thus results into an increase in FRET efficiency from 0.77 to 0.90 for native HSA (Supplementary Information Table 1 to 7). As evident from Figure 2C, like Dextran 40, the increase in distance between the FRET pair as a function of the denaturant also exhibits a certain degree of cooperativity, the same remaining almost invariant to the concentration of Dextran 70 used. In case of Dextran 6, however, distinct changes can be seen. At 25 g/L, the efficiency of energy transfer is same as that of buffer showing a value of 0.65, implying no effect on the interdomain distance at this concentration of the crowding agent (Figure 2D and Figure 3). Another striking aspect of the domain movement observed in presence of Dextran 6 is that, unlike Dextran 70, the distance between the domains increases as one increases the Dextran 6 concentration from 25 g/L to 175 g/L, the latter being the highest concentration of any crowder used in this study. Moreover, in presence of Dextran 6, the domains show only slight relative movements over the range of urea concentrations the protein was exposed to, suggesting that this macromolecular crowder imposes a severe restriction on the conformational subspace of HSA thereby hardly allowing it much freedom to move (Figure 2B).

To further analyse the aforesaid domain movement in a better way, we have plotted the variation of the Trp-Ac distance with increasing crowder concentration as a function of urea in Figure 3. Indeed, as referred to above, when Dextran 40 was used as the crowding agent the distance between FRET pair remains almost constant beyond 50 g/L, this feature being preserved at all concentrations of the denaturant. For Dextran 70 at the lower to moderate denaturant concentrations (from 0 M to 5 M) there was a steady decrease in interdomain separation ~ 26 Å to ~ 18 Å for 0-1 M urea and ~ 26 Å to ~ 22 Å for 2-3 M urea. At the higher urea concentrations of 7 M and 8 M, the decrease in distance between Trp and Ac became distinctly biphasic, signifying the presence of intermediate states. On the other hand, Dextran 6 not only induces an increase in the Trp-Ac distance with increase in its concentration but also brings about the maximum interdomain separation, amongst the crowding agents used, in particular at the higher crowder concentrations (g/L). Moreover, it is evident (Supplementary Information Tables 1-7) that the maximum modulation of Trp-Ac FRET pair distance occurred in presence of the higher molecular weight crowders (Dextran 70 and Dextran 40).

Thermodynamic analyses: Correlation between domain separation and global unfolding

Thermodynamic analyses for the urea denaturation profiles were performed using two different parameters and assuming a two-state unfolding process: (i) Using the changes in the interdomain distances as a signature (as shown in Figure 2) and (ii) the changes in Trp-214 emission (SI Figure 2) that has commonly been employed for protein thermodynamics. The profiles were fit to equation 5 where in case of the distance changes, F should be read as r, the distance between the Trp-Ac FRET pair, all others remaining the same. The extracted thermodynamic parameters have been shown in Tables 1 (from Trp emission) and 2 (from changes in r). ΔG as a function of crowder concentration for both the global unfolding and r-based changes in crowded media has

been plotted in Figure 4. All the three crowding agents show an increase in the free energy of unfolding (Figure 4A) when monitoring the global changes, implying that these stabilize HSA towards chemical denaturation. Dextran 6 had the largest change in the unfolding free energy followed by Dextran 70. Interestingly, the increase in $D_{1/2}$ was almost the same (midpoint of urea denaturation increased by ~0.5 M urea at 175 g/L crowder) in all the three cases, meaning that the change in ΔG°_{u} was countered by a parallel increase in the value of m_G. The latter (m_G) has often been associated with the degree of exposure of residues, that is, the solvent accessible surface area (SASA)[23]. In other words, larger stabilization to urea denaturation was brought about at the expense of greater exposure of protein residues during this process. Thus it goes without saying that Dextran 6 gave rise to the most solvent exposure (in this case the solvent being composed of varying concentrations of the respective crowding agents), this picture being consistent with the increased separation of the two domains.

The FRET modulation profile was also fitted using the same equation and the results were further compared with the global unfolding parameters. As evident from Table 2, in presence of Dextran 40, beyond 50 g/L the unfolding free energy (Figure 4B) and degree of solvent exposure remained almost the same. This is in good agreement with the changes in interdomain distance as plotted in Figure 2, wherein beyond 50 g/L, the curves were all quite close to each other, that is, almost superimposing. Similar to that observed for the global unfolding profiles, Dextran 70 again had the effect of increasing the free energy of domain separation with increase in concentration of the crowder as evident from Figure 4B. Given the fact that the change in separation of the Trp-Ac FRET pair might not be providing us a complete picture of the unfolding of HSA, however surprising qualitative similarities could be observed between the thermodynamic parameters extracted from the non-linear least squares fitting of the two profiles.

For example, apart from the steadily increasing free energy of unfolding for both, the m_G values for Dextran 70 and Dextran 40 from the FRET data are quite comparable to those obtained from the global Trp changes, indicating that the exposure during the global unfolding was mostly arising from the relative movements of these two domains of HSA. Unfortunately a similar comparison could not be carried out for Dextran 6 since in the latter the domains showed very little relative movement.

From Figure 3, both the higher molecular weight crowders of Dextran 40 and Dextran 70 show a decrease in interdomain distance as the crowder concentration is increased. However, as also has been referred to before, beyond 50 g/L, the FRET pair separation remains almost constant for Dextran 40 as opposed to a gradual decrease observed in the case of Dextran 70. Based on the general idea of excluded volume, Dextran 40 having a smaller average molecular weight, has almost twice the number of molecules per unit weight of crowder than that of Dextran 70 and hence a higher packing density. Thus, the former is expected to bring about greater compaction of the protein with respect to the two domains. We hypothesize that based on the higher number density, Dextran 40 has the ability to solvate the protein in a more effective way. At 50 g/L, the primary layer of Dextran 40 around HSA is already well-formed and hence further increase of concentration of the crowding agent has no further impact on the protein. In other words, akin to hydration effects wherein the primary solvation layer is mostly responsible for the effects on the biomolecule, for Dextran 40, the crowder molecules beyond the first layer remain essentially non-perturbing. Thus beyond 50 g/L, the number of Dextran 40 molecules in the immediate proximity of HSA remains unchanged, thereby giving rise to an almost constant interdomain distance. In case of Dextran 70 the packing efficiency is less based on the lesser number density and hence the solvation layer remains partially filled. As a result increasing the concentration of

Dextran 70 brings about a progressive increase in the number of crowder molecules in the vicinity of HSA thereby causing a gradual decrease in the FRET distance.

The distance changes in presence of Dextran 6 provide a very interesting outlook on how this crowder exerts its influence on the protein. Dextran 6, having the least molecular weight among the crowding agents used was expected to provide the maximum excluded volume effect and thereby resulting in the highest efficiency of energy transfer. However quite contrary to our expectations, it gives rise to the least efficiency, revealing that the manner in which the effect of crowding is experienced by HSA is quite different. The most notable and surprising profile was obtained at the low denaturant concentration (0 M to 3 M Urea) where the Trp-Ac distance started from a high value and remained almost constant over the entire range of urea concentrations, thereby revealing little or no effect on the FRET pair separation in presence of this crowder. However, at high denaturant concentrations (6-8 M urea), Dextran 6 (at the lower concentration of 25 and 50 g/L) induces appreciable reduction in the Trp-Ac distance to the extent that the donor-acceptor pair approach even greater proximity than that of Dextran 40 and Dextran. Beyond these concentrations the Trp- Ac distance increases gradually. Moreover from Figure 4 it was observed that $\Delta G^{\circ}_{\text{global unfolding}}$ increased steadily with increasing concentration of Dextran 6 concentration but on the contrary only a moderate change was observed between domain I and II distance (Figure 2D). However the fact that such a large difference in ΔG° obtained from the global unfolding data is not reflected at all in the interdomain separation profile implies that some other part of the HSA that is spectroscopically silent in our present study, is undergoing appreciable modulation in conformation. This observation thus suggests that the excluded volume exerted can be quite unsymmetrical in nature with respect to the domain alignment such that different parts of this multidomain protein are affected differently in

presence of the crowding agent (Scheme 1). (We would like to clarify at this point that since urea could not bring about complete denaturation, attempts were made to follow the unfolding profile in presence of the more potent guanidinium hydrochloride (GdmCl). However when the macromolecular crowders were present, these along with the high concentrations of GdmCl gave rise to significant scattering at 295 nm excitation that resulted in highly varying and irreproducible FRET efficiencies).

Thermal denaturation of chemically unfolded HSA

HSA does not undergo complete unfolding in presence of 8 M urea; thus a significant portion of the unfolded basin remains inaccessible (i.e. not probed) during our measurements. Hence, in order to probe the same, we have performed thermal denaturation studies on the chemically unfolded HSA and Ac-HSA in buffer and the dextran based crowding agents, with the concentration of the latter being increased from 50g/L to 175 g/L. The urea concentrations chosen were 2 M, 4 M, 6 M and 8 M such that it spanned the entire denaturation range while the temperature was varied from 10 – 90 °C.

FRET changes in the unfolded basin

In buffer, increase of temperature at defined urea concentrations brought about increase in separation between the FRET pair. In absence of the chemical denaturant, the interdomain (domain I & II) distance between the FRET pair undergoes the largest increase from ~ 26.5 Å to ~ 28.5 Å between the temperature range of 40-70 °C coinciding with previously reported thermal denaturation midpoint (T_m) for the protein to be around 60 °C (Figure 5). For native HSA and 2 M urea, the thermal transitions as monitored by the distance changes, were almost superimposable on each other, while at 4 M urea, the change in *r* not only becomes sharper but

shifts to a lower T_m value of ~ 50 °C. Such a drop in the midpoint of thermal denaturation is expected due to the initial destabilization of native HSA in presence of 4 M urea leading to earlier onset of protein unfolding. At 6 M and 8 M urea, the extent of denaturation is large enough that the efficiency of energy transfer is low thereby giving rise to increased separation between the Trp and Ac moieties. When the denaturant concentration is low (0 – 4 M Urea) maximum change in Trp-Ac FRET pair is observed as evident from Table 4. Far UV CD also again confirmed the shift of T_m towards lower value with increasing denaturant concentration (Supplementary Information Figure 4).

Similar measurements were also carried out in presence of the dextran based crowders (Figures 6- 8). In case of 50 g/L Dextran 40 (Figure 6A) at 0 M urea there was a sharp increase in Trp-Ac distance around 50- 70 °C (~ 22 Å to ~ 28 Å) beyond which it remained almost constant (~ 29 Å). This increase in interdomain separation is further shifted towards lower temperature for 4 M urea (40- 60 °C) as evident from Figure 6A. At a high concentration of urea (6 M) the thermal denaturation takes place over a broad range of temperatures. At a very high concentration (8 M) of urea no such thermal denaturation was observed. In other words, the transitions profiles based on the FRET pair distance are very similar to that observed in buffer, except that the extent of coopertaivity shown at the concentrations of 0, 2 and 4 M urea are more than could be seen in its absence. As evident from Figure 6B – F the overall trends in domain separation was very similar to that observed for 50 g/L, in conformity with that observed in case of chemical denaturation, wherein beyond this concentration of Dextran 40, there was no further change in the Trp-Ac separation.

The FRET modulation with temperature in case of Dextran 70 is quite different than that of Dextran 40. The most evident difference as can be seen from Figure 7A is with regards to the

nature of the transitions and the actual change in distance that the FRET pair can undergo in presence of this high molecular weight crowder. Dextran 70 brings about a dramatic decrease in coopertaivity of the domain movement, while at 4 M urea and beyond, the transitions become quite non-cooperative. Moreover, except at the lower denaturant concentrations wherein Δr is ~5.4 Å (Table 4), the interdomain distance hardly shows any changes at the higher urea values. From Figure 7B – F it is evident that for all the crowder concentrations the local unfolding profile is following a similar trend. Indeed, irrespective of the crowder and denaturant concentration it is clear from the plots (Figure 8) that Dextran 70 imposes a major restriction on the conformational space available to domains I and II under conditions of thermal denaturation.

The lowest molecular weight crowder used in this study, Dextran 6, brings about a very interesting set of FRET distance changes, with the manner in which these modulations occurs showing stark dependence on the crowder concentration, an obvious deviation from what has been encountered thus far for the other two dextran based crowding agents (Figure 8). At 50 g/L Dextran 6, the Trp-Ac distance shows a cooperative unfolding profile with increasing temperature at low concentration of denaturant (Figure 9 A). At high concentration of urea i.e. 6 M and 8 M the interdomain distance shows a steady increase from ~ 27 Å to ~ 31 Å and ~ 28 Å to ~ 32 Å respectively. At 100 g/L, the trends are observed to have become more non-cooperative with the cooperativity being restored at the higher concentrations of Dextran 6. That the domain movement in presence of Dextran 6 is quite different from the other crowders is also noticeable from the fact that even for 8 M urea, the distance change between Trp and Ac can show signs of cooperativity.

Temperature plays a crucial role in mediating the interaction pattern of HSA with the crowder molecules. For the protein, the effect of a crowder molecule, if assumed to exert its influence

through exclusive hard-core repulsions only, is dependent on the packing density, that is, the presence (or absence) of interstitial voids. Dextran 6 is the smallest macromolecular crowder considered in our study, thereby having the maximum packing density and minimum interstitial void. In this regard, it was expected to bring about the smallest change in domain separation during thermal unfolding. However, as discussed before, Dextran 70, having the least packing density, resisted thermal unfolding of HSA the most. This suggests that the effect of crowding is not entirely due to excluded volume arising from the hard-sphere scenario based on pure mutual impenetrability of the macromolecules involved. Moreover, in an attempt to point out the general trends obtained from the thermal unfolding data, the following points are worth taking note of: (i) $\Delta r = r_{90} - r_{10}$, where r_{90} and r_{10} are the Trp-Ac distances at 90 °C and 10 °C respectively, for any given crowder concentration, peaks at 2 M urea and thereafter follows a decrease as the urea concentration is increased (Table 4) (ii) Increase in temperature leads to an increase in the interdomain distance, albeit to different extents, depending on the crowder involved (iii) The distance change profiles in 6 M and 8 M urea are primarily non-co-operative as can be explained based on the fact that the protein has already undergone major disruption of its stabilizing forces, that is, is already quite denatured.

Summary

The precise manner in which multidomain proteins fold or unfold is a matter of great interest and of equal complexity. Of particular challenge is the task to decipher the extent of cooperativity or the absence of the same that different domains exhibit, as the protein meanders through the conformational subspace, in response to any external perturbation. Added to this seemingly difficult problem of visualization, is the excluded volume phenomenon that different

macromolecular crowders exert on the different domains and hence the protein as a whole. Human serum albumin in that respect is an apt choice for studying domain response in presence of macromolecular crowders because of the following structural and functional aspects that the protein exhibits: (i) it has three domains of which domain I has a free cysteine that can be labelled with an external fluorophore (ii) there is only one Trp residue in the protein and is located in domain II (iii) HSA, being a protein with well-defined drug binding sites and a transporter of fatty acids, has been used extensively in drug binding studies. While the first two points are advantageous in probing interdomain distances, the last one is also extremely important with regards to the fact that binding of fatty acids can often involve large scale domain movements. Indeed, how the multidomain protein behaves or functions in the crowded milieu depends significantly on the extent to which such motions get affected.

Our data suggest that the crowding agents used, depending on their shape and size can have significant influence on the way the individual domains (domains I and II) respond to the effect of reduced space for conformational sampling. FRET studies have revealed that the relative movement between domains I and II can show dramatic variations. Below we summarise few of our significant findings so as to provide a clearer picture of how these synthetic crowders might have had influenced the mulitdomain protein:

(i) During chemical denaturation both in presence of Dextran 70 and Dextran 40 larger Δr (0 to 8 M) values (Table 3) are observed as compared to that of buffer while Dextran 6 shows lesser Δr. At this point it is worth spending some time thinking about the implications of these results. On one hand, we have shown that Dextran 6 provides an elongation along the Trp-Ac FRET axis unlike the other crowders (Scheme 1). However, on the other hand, it is also true that Dextran 6 induces the smallest change in Δr. In other words, these show that Dextran 6 does exude the

maximum excluded volume wherein it provides the stiffest resistance to domain separation. Moreover, the fact that this crowding agent forces the two domains farther apart is also an evidence of the manner in which the protein adjusts and thereafter responds to increased confining pressure from the surrounding Dextran 6 molecules, an aspect that might very well be related to the intrinsic compressibility of the protein.

(ii) For the thermal denaturation profiles, Dextran 70 shows the least variation in Δr (10 – 90 °C) followed by Dextran 6 and then by Dextran 40 (Table 4). This reversal of the effect in domain separation in the temperature dependent experiments for Dextran 70 is an aspect that suggests that some other factors besides excluded volume have to be taken into consideration to provide a reliable picture of its effect on HSA (discussed below).

(iii) Irrespective of the mode of denaturation, that is, either chemical or thermal, in presence of Dextran 40, Δr is the maximum. Moreover, the manner in which this crowder affects HSA is such that it brings about the largest distance change within 50 g/L beyond which no further effect of increased crowder concentration is visible.

(iv) The $\Delta G°_u$ values obtained from global unfolding are consistently higher than those from FRET modulation arises from the fact that the latter shows the extent of unfolding with respect to the interdomain separation only, as we have mentioned before. However the similarity of these values reveals that not only does the movement of domains I and II capture a significant portion of the urea-induced denaturation of HSA, but also that the interdomain distance, or more rigorously, the Trp-Ac distance, *r,* is also a valid signature of the unfolding of the protein unfolding. Such an observation is of tremendous importance in the folding mechanism of multidomain proteins wherein instead of the commonly accepted sequential folding pathway[25], these uncoupled domains can fold by themselves in a parallel fashion (simultaneously) thereby

minimizing the search on the erstwhile multidimensional energy landscape and hence speeding up the folding of the protein.

The effects of cosolutes (small or macromolecular) or additives on conformations of bio-macromolecules have always been a matter of intense discussion[27-30]. From the well-established Asakura-Oosawa model (AOM)[31-32] that modelled the influence of cosolutes using steric effects (and hence ascribed the observations to predominantly entropic considerations) to the incorporation of soft repulsions/interactions, the latter bringing in the enthalpic component, several modifications and advancements have been made over the years to understand the underlying interactions more comprehensively. Recent papers by Harries and coworkers have discussed at length the presence of depletion forces originating from enthalpy effects, in particular, for small molecular crowders or osmolytes[33-35]. Purely based on steric aspects (AOM), the free energy change involved in protein (un)folding should show a linear variation with respect to the cosolute concentration. In this regard, the variation of $\Delta G°_u$ for Dextran 6 (inset to Figure 4A) fits this prediction showing that this macromolecular crowding agent, having the least average molecular weight amongst the crowders used in this study, has entropy as the primary factor behind its excluded volume effect. Indeed this explains the fact that for both chemical and thermal denaturation, Dextran 6 shows a very small variation in Δr, with the latter being more in case of the thermal profiles probably due to temperature induced fluctuations in the absolute packing density of the crowding agent. For Dextran 70, the near sigmoidal dependence of $\Delta G°_u$ on crowder concentration, suggests that the manner in which it influences HSA has additional components beyond entropic depletion forces. This trend observed for the chemical denaturation induced HSA conformational ensembles also points towards a cooperative arrangement of the Dextran 70 molecules around the protein. Recent studies have suggested that high molecular

weight crowders along with entropic depletion forces can also exhibit enthalpy driven effects[33-37]. The fact that even at concentrations as low as 25 g/L Dextran 70, wherein the excluded volume effects due to steric reasons should be at its minimum, it is able to bring about significant reduction in the interdomain separation (Figure 3) suggests the presence of appreciable soft interactions between the crowder and the serum protein. A similar presence of enthalpy dominated soft potential is also observed for Dextran 40; however for the latter, the fact that the interdomain separation remains almost constant beyond the concentration of 50 g/L (Figure 3) implies that its arrangement around the protein is quite different than that of Dextran 70. Moreover, Dextran 40 shows the maximum interdomain separation for both the modes of denaturation, revealing that neither does it have enough enthalpic effects to exhibit significant soft interactions nor is its entropy based excluded volume effect prevalent to a great extent. Furthermore as one move from Dextran 70 to Dextran 6, we go from a decrease in interdomain separation, to one that remains constant in presence of Dextran 40 followed by a case of increase in domain separation for Dextran 6. These taken together suggest a distinct crossover in the nature of depletion forces operational for the polymeric crowding agents, with effects of Dextran 6 being mainly entropic in nature, Dextran 70 showing evidence of both entropic and appreciable enthalpic contributions, while Dextran 40 having characteristics of both Dextran 70 and Dextran 6, it being midway with regards to the average molecular weight of the other two crowders.

Recent surge in research activities trying to understand the intricacies of macromolecular crowding (in an effort to understand the cellular milieu) has revealed that apart from the well-established excluded volume effects, macromolecular crowders can also exhibit soft interactions with the test protein molecules. Inside the cellular environment, the crowded milieu is composed of many different macromolecules, with proteins being one of the major components. Hence it is

obvious that surface charges of proteins will play a significant role in defining the extent of electrostatic contribution that these crowder proteins exhibit with the biological molecule under investigation. Pielak and coworkers have shown that the effect of reconstituted cytosol on the globular protein Chymotrypsin Inhibitor 2 (CI2) is governed mostly by weak non-specific interactions of the host proteins present[38-39]. While evidence with regards to soft interactions of synthetic macromolecular crowders on proteins has been hard to come by, a couple of recent reports have revealed that Ficoll 70 and Dextran 40 behave quite similar to their monomeric building blocks of sucrose and glucose respectively, and these too exert their influence via an enthalpic stabilization mechanism[40]. In this regard our study shows a similar presence of soft interactions for Dextran 40 and Dextran 70, that is, the high molecular weight crowders. Moreover, the fact that Dextran 70 shows the least Δr for the thermal denaturation studies, might arise from the enhancement of its soft interactions under these conditions, probably hinting at the existence of appreciable dispersion forces between the polymeric Dextran 70 molecules and the serum protein.

Conclusions

Domain interfaces in multidomain proteins have been hypothesized to play a critical role in the overall folding of the multidomain protein. If interfaces are predominantly hydrophobic, that is, if they present 'a sticky interface[41], proper masking of the same by the other domains is necessary such that the protein does not become prone to aggregation. HSA is an avid transporter of fatty acids having as many as seven fatty acid binding sites. Not only does the binding of some of these involve large-scale domain movements, but also some of the fatty acids have been shown to bind at the domain interfaces. Crystal structure of myristate bound HSA reveals

significant angular displacement of domain I (relative to domain II) and opening up of the central crevice with increment of the molecular width of the protein upon binding of the fatty acid[42]. The modified inter domain distance(s) in presence of macromolecular crowders suggests that such binding sites and associated local protein movements can get appreciably modulated in presence of the macromolecular crowders thereby affecting the fatty acid binding and transport. The combination of protein crowder soft hydrophobic interaction (for higher molecular weight crowder) with the asymmetrical nature of the packing density (as mentioned before) for Dextran 6 introduces an interesting bias in the energy landscape of this protein, an aspect that needs to be investigated further using suitable kinetic methods. Indeed it remains to be seen to what extent such domain compaction and expansion by different macromolecular crowders can modulate the so-called rugged energy landscape, thereby affecting kinetic pathways and intermediates and even possibly switching between alternate folding mechanisms, with the latter depending on the type of excluded volume the mutlidomain protein experiences *en route* to the native state.

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Supporting Information

The supporting information contains Efficiency and interdomain (domains I and II) distances during chemical denaturation of HSA in absence of any crowding agents and 25 g/L of macromolecular crowder (Table 1), 50 g/L (Table 2), 75 g/L (Table 3), 100 g/L (Table 4), 125 g/L (Table 5), 150 g/L (Table 6) and 175 g/L (Table 7). Overlap between normalised absorption spectrum of acceptor (Ac) and emission spectrum of donor (Trp) in phosphate buffer was shown in Figure 1 and Normalised emission spectra of Trp in (A) Dextran 40, (B) Dextran 70 and (C) Dextran 6 with increasing concentration of crowders was shown in Figure 2 A-C. Figure 3 contains the representative thermodynamic fits of chemical denaturation (as a function of Trp-Ac distance) of HSA in buffer and various crowded media. Normalized thermal denaturation profile of HSA as monitored using CD at different urea concentrations in buffer was shown in Figure 4.

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Table 1: Thermodynamic parameters for urea induced global unfolding of HSA in buffer and different crowded media based on Trp fluorescence changes.

Crowder	Crowder concentration (g/L)	$\Delta G°_{\text{global unfolding}}$ (kJ mol $^{-1}$)	$-m_G$ [kJ/(mol.M)]	$D_{1/2}$ (M)
DEXTRAN 40	0	11.4	2.3	4.9
	25	12.0	2.5	4.8
	50	14.5	2.8	5.2
	75	14.6	2.8	5.2
	100	15.0	2.8	5.3
	125	15.7	2.9	5.3
	150	16.6	3.1	5.4
	175	16.8	3.1	5.3
DEXTRAN 70	0	11.4	2.3	4.9
	25	12.5	2.5	5.0
	50	12.8	2.5	5.1
	75	16.0	3.1	5.2
	100	19.6	3.8	5.2
	125	20.0	3.8	5.3
	150	21.0	3.8	5.5
	175	nd	nd	nd
DEXTRAN 6	0	11.4	2.3	4.9
	25	12.5	2.5	5.0
	50	16.0	3.1	5.1
	75	19.6	3.8	5.1
	100	20.0	3.8	5.2
	125	22.0	4.1	5.3
	150	23.4	4.3	5.4
	175	26.0	4.8	5.4

Table 2: Thermodynamic parameters for Urea induced domain separation of HSA (obtained from interdomain distance between domain I and II)

Crowder	Crowder concentration (g/L)	$\Delta G°_{\text{domain separation}}$ (kJ mol $^{-1}$)	$-m_G$ [kJ/(mol.M)]	$D_{1/2}$ (M)
DEXTRAN 40	0	11.1	2.3	4.8
	25	12.0	2.5	4.8
	50	14.5	2.8	5.2
	75	14.6	2.8	5.2
	100	15.0	2.8	5.3
	125	14.8	2.9	5.1
	150	15.4	3.0	5.1
	175	14.3	2.8	5.0
DEXTRAN 70	0	11.1	2.3	4.8
	25	12.5	2.51	5.0
	50	12.8	2.5	5.1
	75	16.0	3.1	5.2
	100	19.6	3.8	5.2
	125	20.0	3.8	5.3
	150	21.0	3.9	5.4
	175	21.5	4.1	5.2

Table 3: Δr (r_{8M}- r_{0M}) as a function of crowder concentration during chemical denaturation of HSA in various crowded media. r_{8M} and r_{0M} are the interdomain (or Trp-Ac) distances at 8 M and 0 M urea respectively.

Buffer (Å)	**[Crowder] (g/L)**	**Dextran 40 (Å)**	**Dextran 70 (Å)**	**Dextran 6 (Å)**
6.3	25	7.8	7.4	2.0
	50	7.8	7.4	1.0
	75	7.5	8.3	0.9
	100	8.7	7.6	1.4
	125	8.1	8.0	2.1
	150	8.2	8.2	3.3
	175	8.4	8.6	4.1

Table 4: Δr ($r_{90} - r_{10}$) as a function of crowder concentration during thermal denaturation of chemically denatured HSA in various crowded media.

Buffer (Å)	Urea (M)	[Crowder] (g/L)	Dextran 40 (Å)	Dextran 70 (Å)	Dextran 6 (Å)
4.8	0 M	0	4.8	4.8	4.8
		50	9.0	1.8	3.1
		75	9.0	1.9	4.2
		100	9.0	2.1	3.8
		125	9.0	2.4	3.5
		150	9.0	2.5	4.9
		175	9.2	2.8	4.9
5.4	2 M	0	5.4	5.4	5.4
		50	9.4	3.2	3.1
		75	9.0	3.1	4.5
		100	9.6	3.2	4.3
		125	9.5	3.6	3.5
		150	9.5	3.7	4.8
		175	9.5	4.1	4.4
4.2	4 M	0	4.2	4.2	4.2
		50	8.4	3.0	3.3
		75	8.4	1.8	4.2
		100	8.2	1.8	4.4
		125	8.2	1.9	3.7
		150	8.3	1.9	4.4
		175	8.2	2.0	4.5
3.9	6 M	0	3.9	3.9	3.9
		50	6.1	3.6	3.8
		75	6.4	2.0	4.1
		100	6.0	1.9	3.6
		125	6.3	1.9	3.4
		150	6.3	1.8	3.5
		175	6.1	1.8	3.5
3.4	8 M	0	3.4	3.4	3.4
		50	4.5	2.5	3.9
		75	5.5	2.9	3.9
		100	4.8	2.4	4.0
		125	5.8	2.2	3.2
		150	5.4	2.2	2.8
		175	4.5	2.1	3.6

Figure Captions:

Figure 1: Schematic representation of secondary structure of HSA is shown and the domains are colour-coded as follows: I, blue; II, green; III, yellow. Cys-34 and Trp-214 are shown in a space-filling representation and colored by atom types (PDB ID: 1E78)[43]. Also shown is the chemical structure of Acrylodan that was covalently ligated to Cys-34.

Figure 2: Comparative plots of interdomain distances (domains I and II) during chemical denaturation of HSA in (A) buffer, (B) Dextran 40, (C) Dextran 70 and (D) Dextran 6.

Figure 3: Variation of interdomain distances with increasing urea induced denaturation (as mentioned in legend) as a function of crowder concentration (g/L).

Figure 4: Variation of ΔG°_{u} as a function of crowder concentration during (A) chemical denaturation (A) and (B) domain separation in various crowded media (as mentioned in legend). Inset of panel (A) shows the linear fit of ΔG°_{u} for Dextran 6.

Figure 5: Comparative plots of interdomain distances (domains I and II) during thermal denaturation of chemically denatured of HSA in absence of any crowding agent.

Figure 6: Comparative plots of interdomain distances (domains I and II) during thermal denaturation of chemically unfolded HSA in Dextran 40 at (A) 50 g/L, (B) 75 g/L, (C) 100 g/L, (D) 125 g/L, (E) 150 g/L, (F) 175 g/L.

Figure 7: Comparative plots of interdomain distances (domains I and II) during thermal denaturation of chemically unfolded HSA in Dextran 70 at (A) 50 g/L, (B) 75 g/L, (C) 100 g/L, (D) 125 g/L, (E) 150 g/L, (F) 175 g/L.

Figure 8: Comparative plots of interdomain distances (domains I and II) during thermal denaturation of chemically unfolded HSA in Dextran 6 at (A) 50 g/L, (B) 75 g/L, (C) 100 g/L, (D) 125 g/L, (E) 150 g/L, (F) 175 g/L.

Figure 1

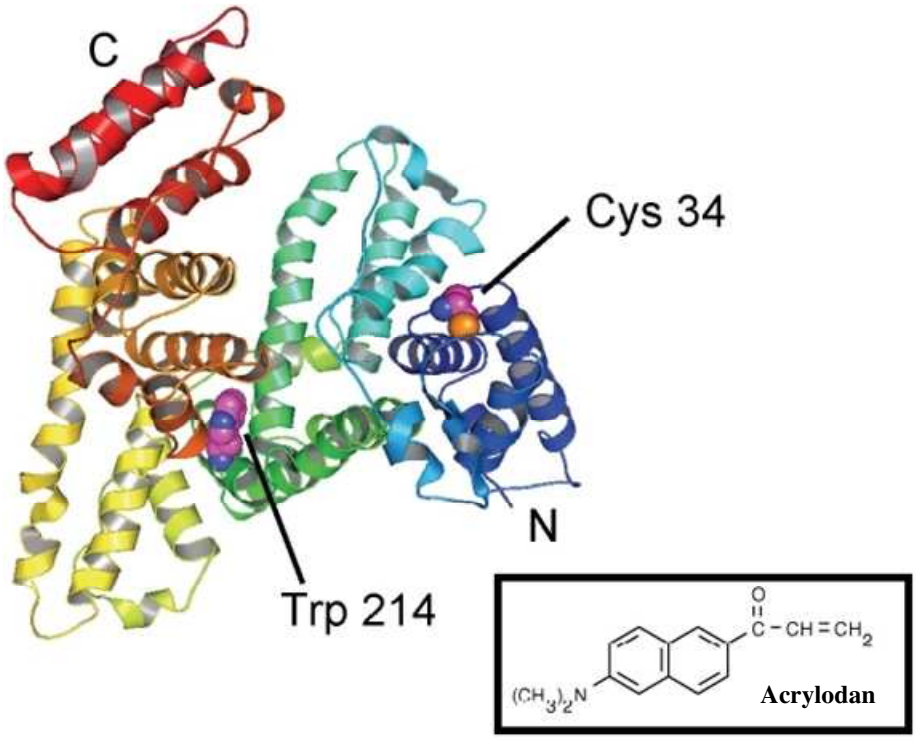


Figure 2

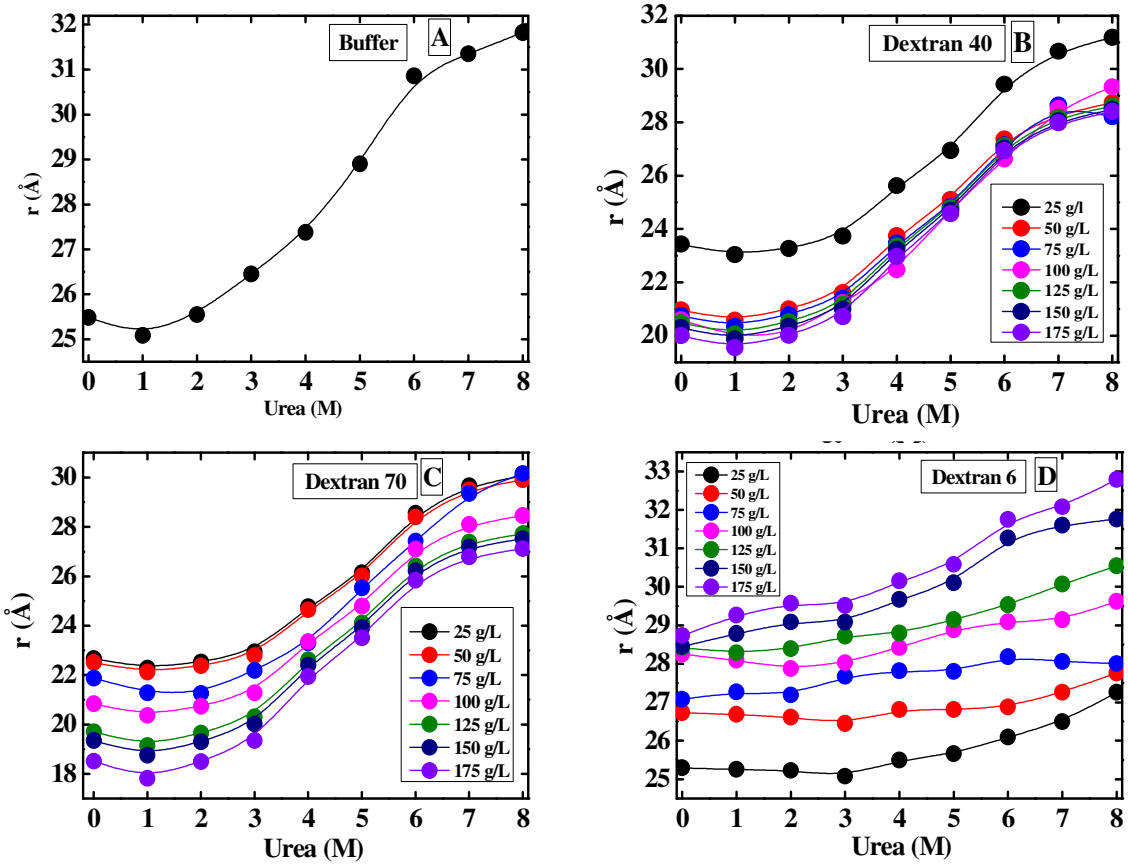
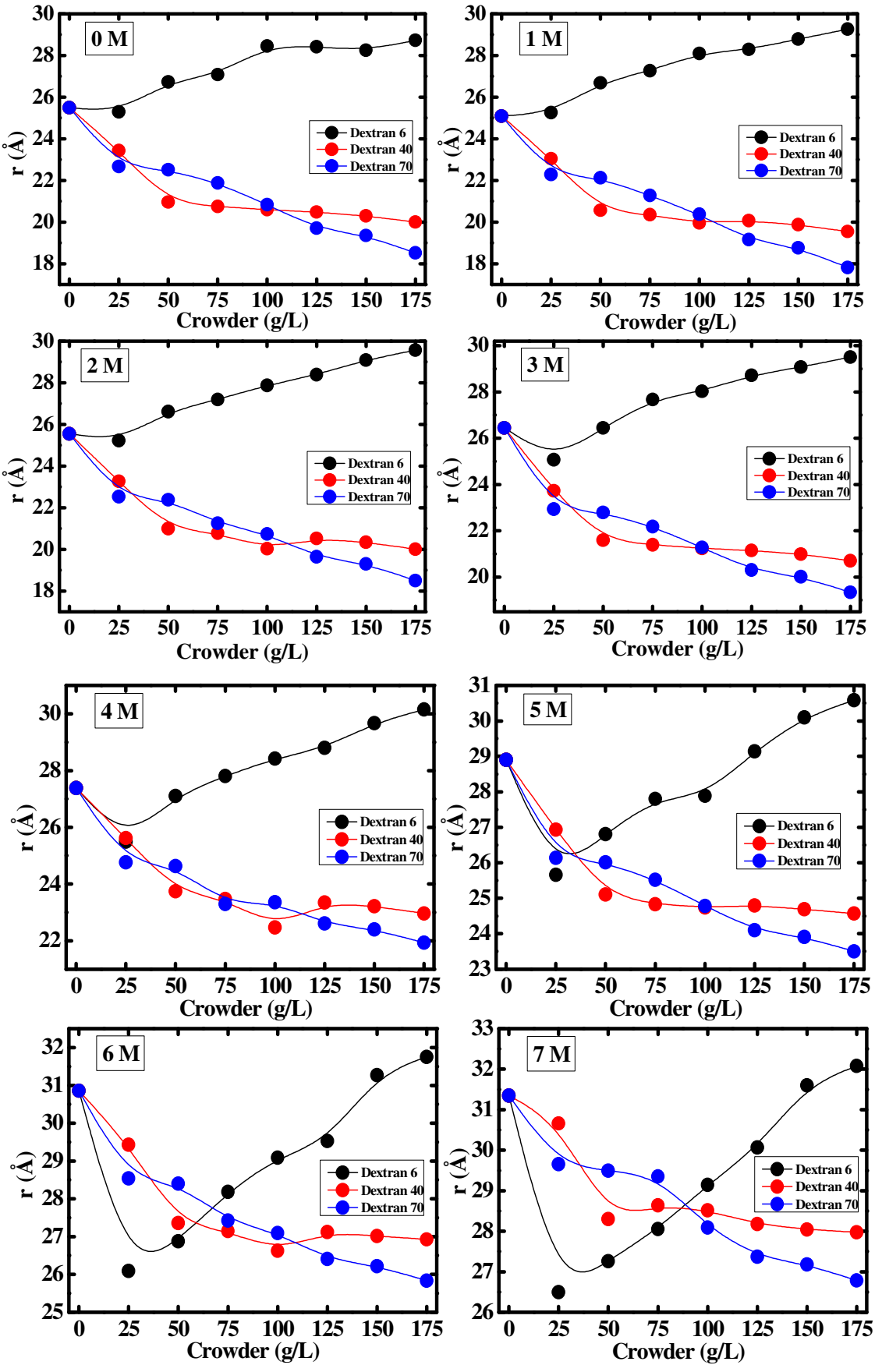


Figure 3



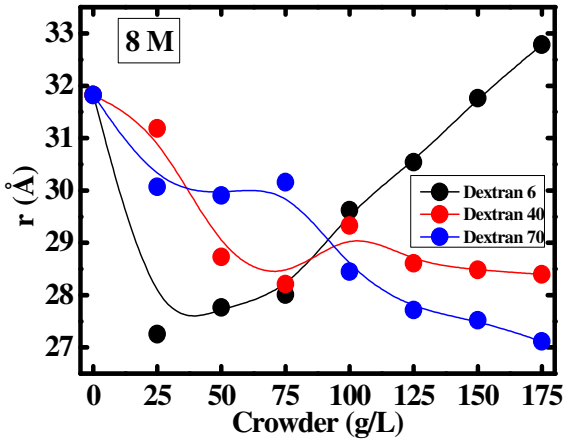


Figure 4

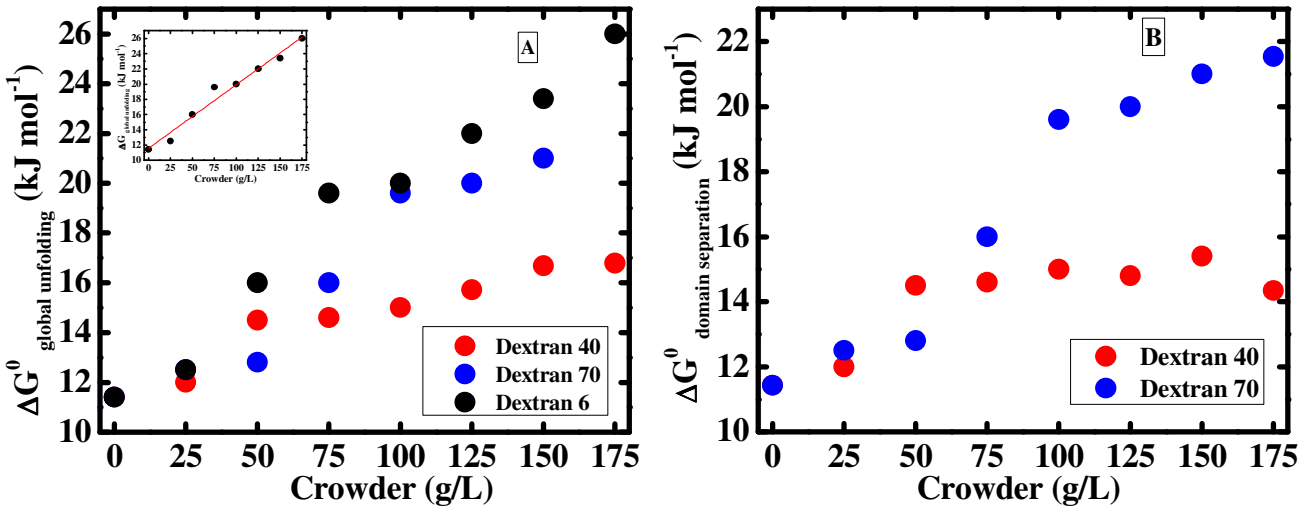


Figure 5

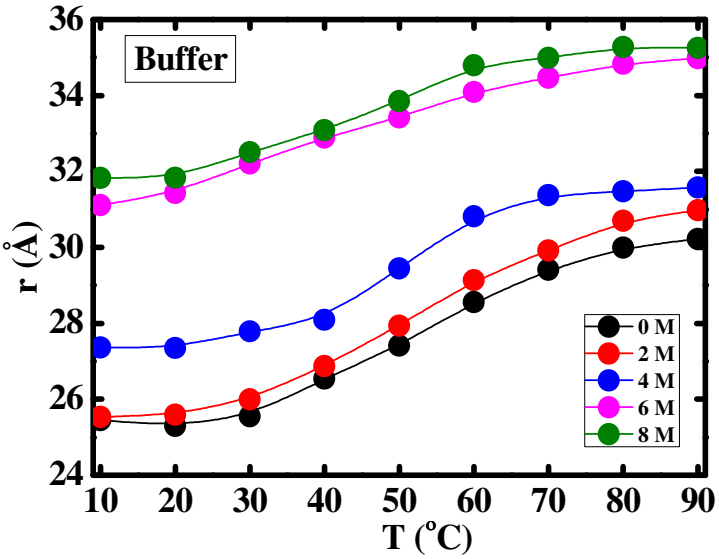


Figure 6

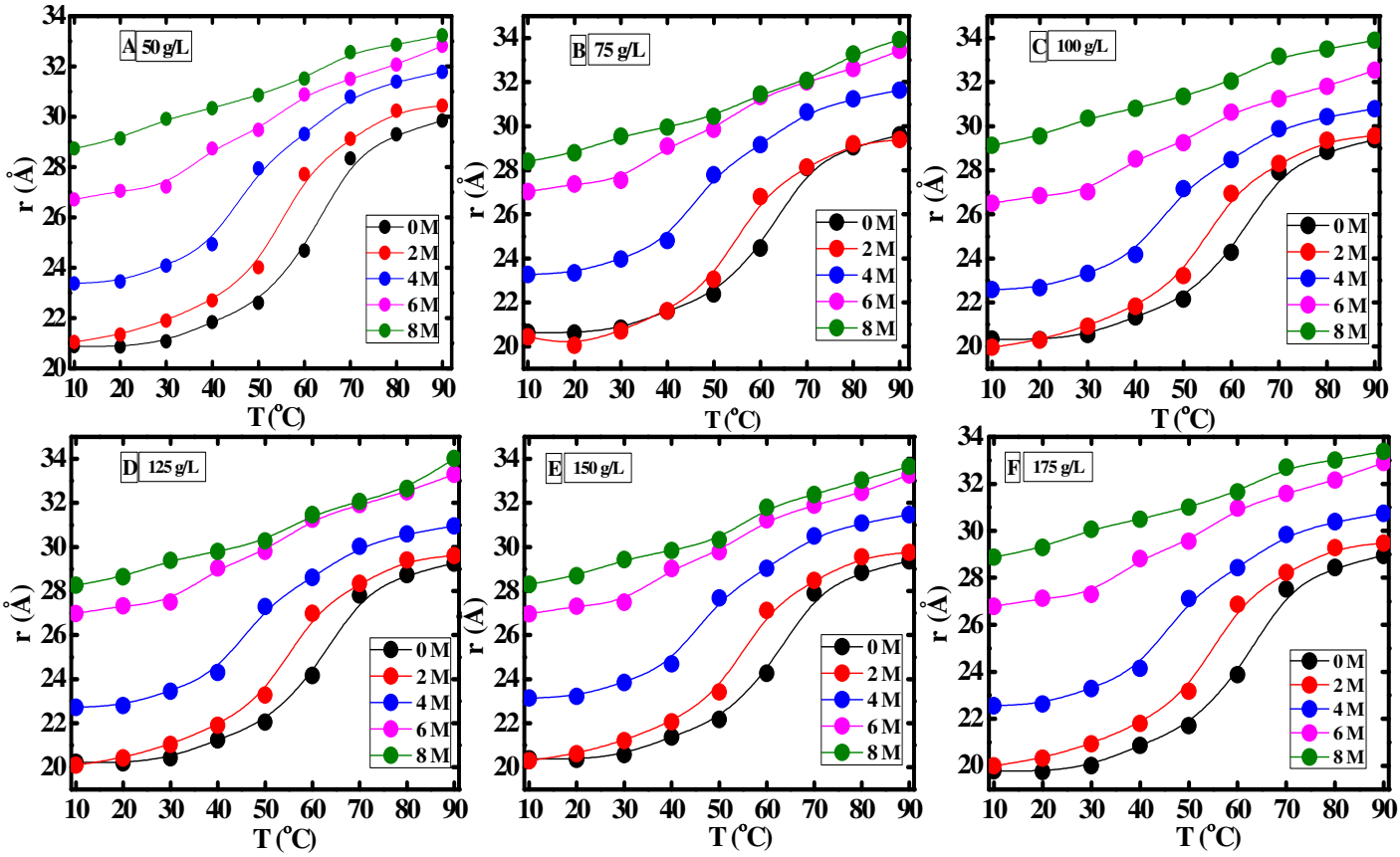


Figure 7

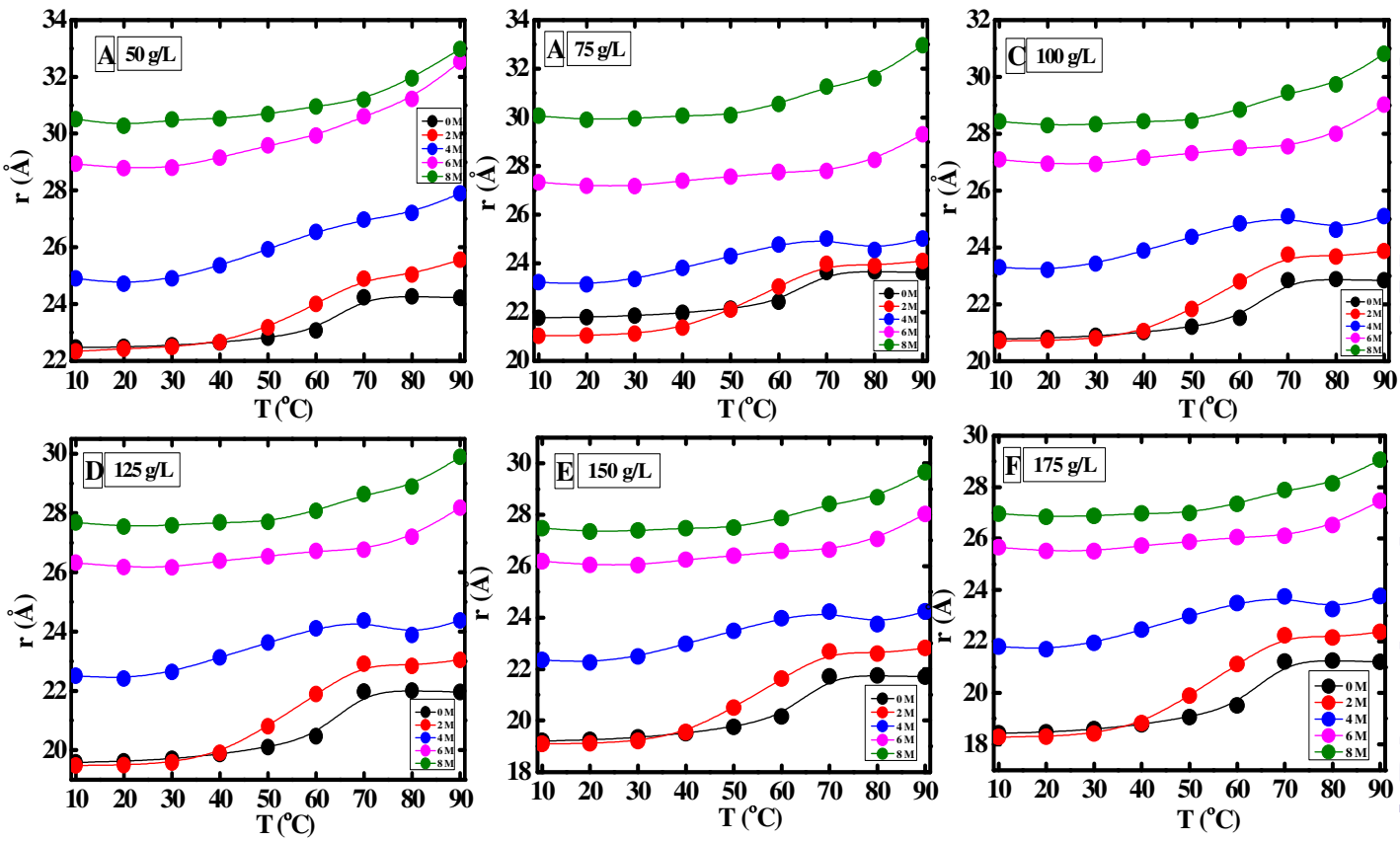
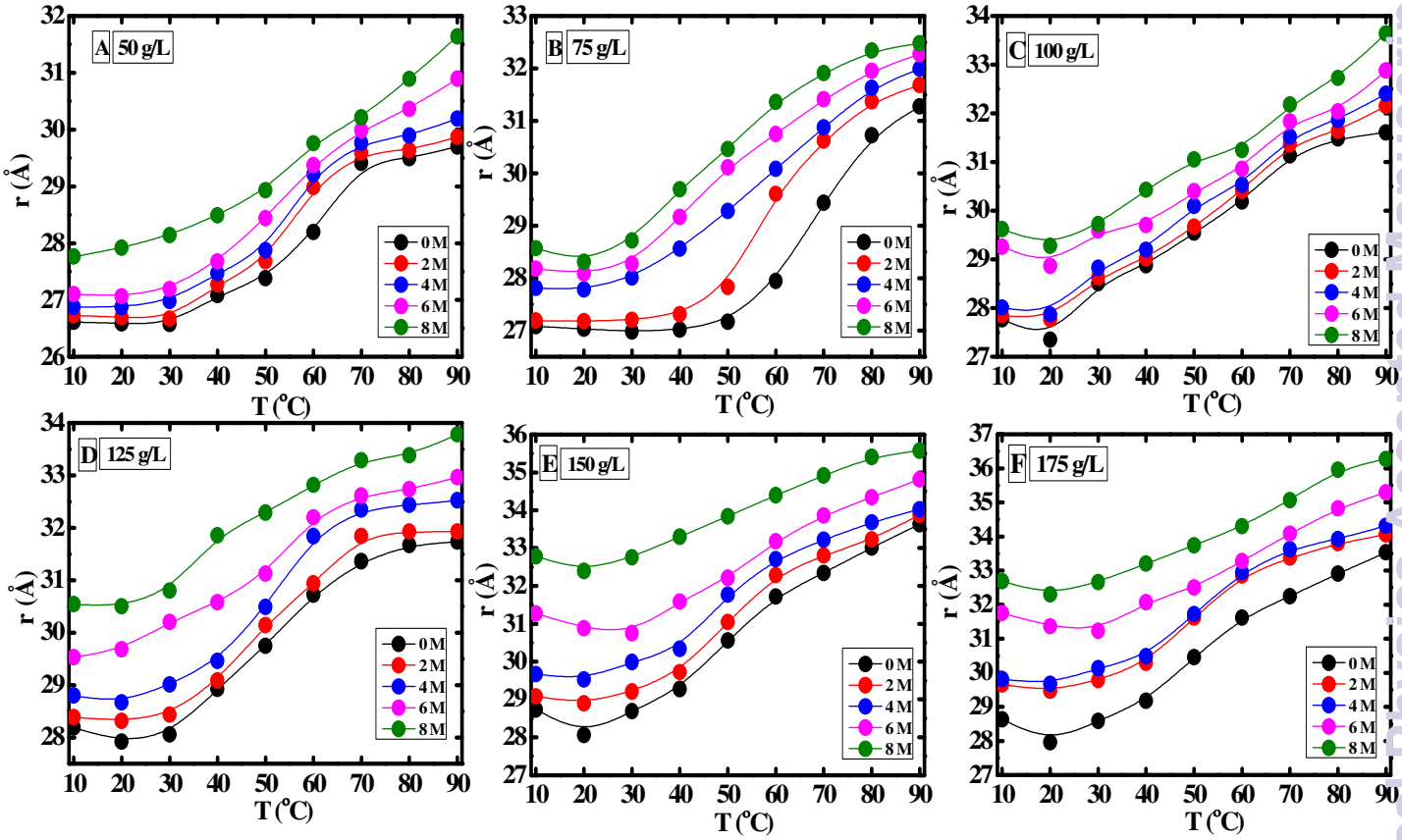
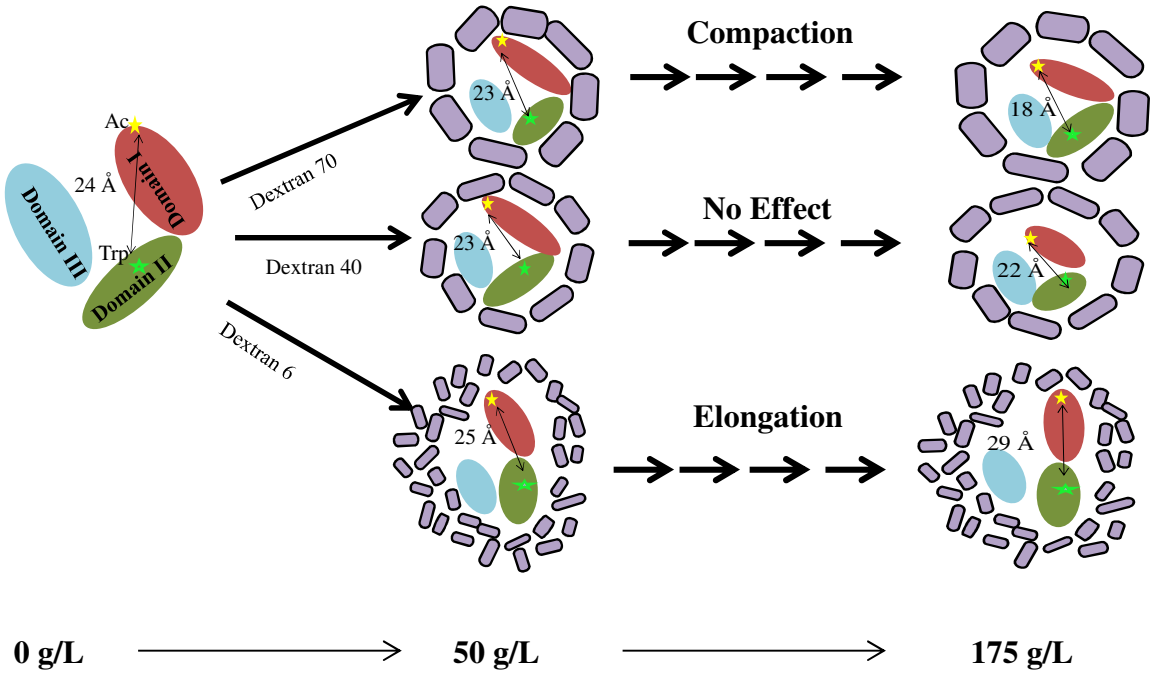


Figure 8





Scheme I: Inter domain distance profiles of native HSA in absence and presence of various crowders. The asymmetry of the excluded volume effect of Dextran 6 has been emphasised.