Hofmeister series salts have an unusual effect on the stability of Toxoplasma gondii Ferredoxin NADP$^+$ Reductase

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Research Article

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Abstract

The ionic interactions play an important role in the stabilization of native conformation of proteins. *Toxoplasma gondii* Ferredoxin NADP+ Reductase (TgFNR) remains stable at pH 4.0 however the modulation of ionic interactions leads to the compaction and non-cooperativity in the folding of domains. To gain further insights into the role of ionic interactions in the stability of TgFNR, stabilized at neutral and acidic pH, salt dependent changes in the structure and thermodynamic stability was investigated. The kosmotropic salts (sodium fluoride and sodium sulphate) appears to induce the biphasic response on the structure and stability TgFNR. At pH about 4.0, the addition of low concentrations of kosmotropic salts significantly perturb the existing native like secondary structure of TgFNR, whereas higher quantities of salt reversed the denaturing impact. This is a one-of-a-kind situation that we are unaware of in any other protein. The urea-induced unfolding of TgFNR in the presence of a low dose of salt (100 mM) drastically affected the protein's thermodynamic stability at neutral pH. Increased salt concentrations, on the other hand, reversed the destabilizing effect, and the salts now behave according to their Hofmeister series ranking. Our findings imply that electrostatic interactions are exceptionally significant in the structure and stability of TgFNR, and the abnormal behaviour of the salts may be mediated by the existence of salt bridges, which stabilizes the native conformation of enzyme.

Introduction

Organization of amino acids with ionizable side chains, impart crucial roles in the determination of structure, function and stability of proteins through the formation of electrostatic interactions. In general, solvent exposed or semi-buried ion-pairs are attracting and have a stabilizing influence on protein stability, whereas buried ion-pairs are repulsive and very destabilizing to proteins. There has been a long running debate towards the contribution of electrostatic interactions in the determination of proteins structure and stability, salt bridges/ion pairs, on the other hand, have been demonstrated to have both a stabilizing and destabilizing effect on proteins, depending on their geometric orientation, structural context, and solvent accessibility [1, 2, 3, 4, 5]. The contribution of electrostatic interactions in the stabilization of protein conformations can be investigated by studying the protein stability studies as a function of variations in the environmental pH and ionic strength. [6, 7, 8]. pH is known to influence the structure and stability of the proteins by altering the net charges. Under extreme pH conditions, the majority of proteins denature because of uniform protonation or deprotonation of ionizable groups resulting enormous repulsion between like charges [9]. Multiple studies indicate that a variety of conformational states are stabilized during acid induced unfolding of the proteins, however those are quite sensitive to salts that affect the electrostatic interactions. Salts exhibit a recurring trend called the Hofmeister series which is more pronounced for anions than cations. Hofmeister series anions affect the stability of proteins following the trend: PO_4^{--} > SO_4^{--} > HPO_4^{--} > F^- > Cl^- >NO_3^- > Br^- > ClO_3^- > I^- > ClO_4^-.With sodium chloride as a neutral salt, anions to the left of the chloride have a higher charge density and are classified as kosmotropes (water structure makers), whereas anions to the right of the chloride have a lower charge density and are classified as chaotropes (water structure breakers). [10].

Though the majority of proteins salt out or salt in, in the same way that Hofmeister series salts do, there are a few exceptions due to ion specificity, hence Hofmeister series universality is still being investigated [8, 12].

TgFNR is an apicoplast localized protein that operates as a general electron switch at the bifurcation step of the many different electron transfer pathways [13, 14]. The enzyme has been proposed as a parasite specific target for the drug development, however the protein's high-resolution structure is yet to be determined. TgFNR has a theoretical isoelectric point of 8.58 and has a considerable number of basic (59) and acidic (49) residues. TgFNR is a monomeric protein with two structural domains: the FAD-binding domain and the $NADP^+$- binding domain. We have demonstrated the importance of electrostatic interactions in the maintenance of conformational stability of enzyme. TgFNR is stabilized in an open conformation with strong interactions exist between FAD and $NADP^+$ binding domain. The folding of the $NADP^+$ binding domain is cooperative, but when ionic interactions are modulated towards an acidic pH (about 4.0), the NADP + binding domain undergoes selective alterations, culminating in a transition to no cooperative folding [15]. We also discovered that the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) exhibited an unusual interaction with TgFNR, in which it not only binds to the enzyme electrostatically but also triggered partial unfolding by changing the enzyme's ionic interaction [16].

To further gain the insights into the role of electrostatic interactions in the stability of TgFNR, we carried out comparative structural and stability studies between neutral and pH 4.0 stabilized conformations of TgFNR. Our results reveal a highly unusual behavior of Hofmeister series salts on the structure and thermodynamic stability of TgFNR as well as indicating that the salt bridges play crucial role in the stabilization of protein at different pH.

Materials & Methods

Most of the chemicals used in the study were purchased from Sigma-Aldrich Chemical Company and were of highest purity available. All chromatographic columns were purchased from Cytiva, USA with the exception of Ni-NTA agarose, which was from Qiagen. Factor Xa protease was purchased from Novagen. Culture media components were purchased from Himedia laboratories, Mumbai, India. Centricon filters were from Millipore. All the buffers were prepared in deionized Milli Q™ water and filtered with 0.22mm filters (Millipore) in filtration device (Tarsons). The pH of solutions was maintained using pH meter with glass electrode (EUTECH Instruments pH 510) calibrated with pH standard buffers obtained from Sigma.

Overproduction and purification of TgFNR

The overproduction and purification of recombinant TgFNR was carried out as described earlier [17] The purified protein was more than 95% pure as evaluated by SDS-PAGE analysis.

Protein quantification

Protein concentration was determined by Bradford method using BSA as a standard.

Fluorescence spectroscopy

Fluorescence spectra were recorded with Perkin-Elmer LS 50B spectroluminescencemeter in a 5mm path length quartz cell. Protein concentration was 3.0 μM for all experiments and the measurements were carried out at 25ºC. For monitoring FAD fluorescence, the excitation wavelength was 445 nm and the spectra were recorded between 450 to 550 nm. Each spectrum was averaged for three scans.

Circular dichroism measurements

CD measurements were made with a Jasco J810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The CD spectra were measured at an enzyme concentration of 3.0 μM in a 0.5 mm cell for far UV at 25ºC. Each spectrum was average of three scans. The obtained values were normalized by subtracting the baseline recorded for the buffer alone under similar conditions. The samples were scanned for in the region 250 – 195 nm for far UV scan.

Chemical denaturation of TgFNR

For pH dependent stability studies, recombinant TgFNR was dialyzed against buffer containing 1mM CGH (Citrate, Glycine, Hepes) and 1 mM DTT at desired pH and unfolding experiments were carried out in the absence and presence of varying concentrations of salts and chemical denaturants. The pH of the solutions at which the measurement was being made was maintained throughout the studies. For unfolding studies, 3 mM TgFNR in 1mM CGH buffer (pH 7.5 or 4.0) was incubated in absence and presence of increasing concentrations of GdnHCl or urea or sodium fluoride or sodium sulfate for 6h at 4°C before spectra were recorded.

For salt dependent urea denaturation, 3 mM TgFNR in 1mM CGH buffer (pH 7.5) was first incubated in desired concentration of NaCl, Na_2SO_4 or NaF for 6h at 4ºC and then urea denaturation was carried out. The urea denaturation curves of TgFNR were obtained by incubating 3 μM proteins in 1 mM CGH buffer, 1 mM DTT (pH 7.5 and 4.0) in the presence/absence of increasing concentration of urea for 6h at 4°C before the measurement were made. The incubation time of 6h was taken as this time was found to be suitable for the system to reach equilibrium under any concentration of urea taken for study.

Data analysis

Urea-induced denaturation curves obtained in the absence or presence of different urea concentrations were used to determine the free energy of stabilization in the absence of denaturants (ΔG_D^{H20}) by linear extrapolation of the ΔG_D values to zero denaturant concentration [18],

$\Delta G_D^{H20} = -RT \ln K$

Where R is the universal gas constant (1.987 cal/deg/mol) and T is temperature in Kelvin. The equilibrium constant, K was calculated from equation

$K = [\theta]_{obs} - [\theta]_F / [\theta]_U - [\theta]_{obs}$

Where $[\theta]_{obs}$ is observed variable parameter and $[\theta]_F$ and $[\theta]_U$ are the values of the variable characteristic of folded (F) and unfolded (U) conformations.

The ΔG_D values were found to be varying linearly with urea concentration, and a least squares analysis was used to fit the data to the equation:

$\Delta G_D = \Delta G_D^{H20} - m\,[urea]$

Where m, is the measure of the dependence of ΔG_D on urea concentration. The denaturant concentration at which $\Delta G_D = 0$, i.e. midpoint of GdnHCl denaturation at any temperature is given by the C_m

$\Delta G_D^{H20} = C_m \times m$

Thermal denaturation

Thermal denaturation of TgFNR (3.0 μM) were monitored by change in molar ellipticity at 222 nm as a function of temperature on a Jasco J810 spectropolarimeter, equipped with peltier temperature controller system. For pH and salt dependent experiments, protein was incubated for 6 h in 10 or 1 mM CGH buffer containing 1 mM DTT at desired pH and salt conditions before measurements were made. The samples were equilibrated at the starting temperature by incubation for half an hour at that temperature. Samples were heated at constant rate of $1^{\circ}C$/min in 1mm path length cell.

Graphing and statistical analysis

Preparation of all the graphs, curve fitting and statistical analysis were carried out either using Origin 7.0.

Results And Discussion

pH dependent stability of TgFNR

Denaturation caused by urea and guanidine hydrochloride (GdnHCl) is often used to determine protein stability [19,20,21]. They act through different mechanisms because GdnHCl is a salt and is predicted to ionise in aqueous solution, whereas urea is not. The Gdn^+ and Cl^- ions could mask the charged residues and screen electrostatic interactions between charged residues on the protein surface. If the charge-charge interactions are repulsive, the Cm value obtained for urea induced denaturation should be lower than that of GdnHCl induced denaturation. If these interactions are attractive in nature, the Cm value obtained for urea driven denaturation under similar conditions should be larger than that obtained for GdnHCl denaturation [21,22,23]. The stability of TgFNR was studied at neutral (pH 7.5) and acidic pH (pH 4.0) by measuring the loss of secondary structure and enhancement of FAD fluorescence as a function of increasing concentration of urea (Fig. 1). Here, with the increase in urea concentration from 0 to 7.5 M, a sigmoidal loss of CD signal and a concomitant enhancement in FAD fluorescence was observed at pH 7.5 and pH 4.0, suggestive of a cooperative transition during the native and unfolded states of TgFNR. However, with the change of pH from pH 7.5 to 4.0, a considerable variation in the Cm value from 4.5M to

1.6M was observed suggesting TgFNR undergoes significant destabilization towards acidic pH. To distinguish between the relative contributions of hydrophobic and ionic interactions, the effect of GdnHCl on the denaturation of pH 4.0 stable conformation of TgFNR was also investigated. In the insets of Fig. 1A and B, the unfolding curves resulting from the loss of CD signal at 222 nm and an increase in FAD fluorescence intensity in the presence of increasing GdnHCl concentrations are given which too are indicative of cooperative transition between folded and unfolded state. Interestingly, the Cm value obtained from GdnHCl unfolding curves was about 0.75 M which is significantly lower than that observed for urea induced unfolding at pH 4.0. Hence, the data clearly indicate that the ionic interactions stabilize the conformation of TgFNR stabilized at pH 4.0, and the electrostatic interactions are attractive in nature.

Salt dependent changes in structural properties of TgFNR

There has been substantial research to probe the role of ionic interactions on the structure and stability of proteins in the presence of different electrolytes [24, 25, 26, 27]. We therefore examined the effect of Hofmeister series anionic salts on the changes in structural properties of TgFNR at pH 4.0 and pH 7.5. We investigated most of the Hofmeister series anionic salts, but only a few of them could be employed due to the chaotropic anions (e.g. Br-, I^-, SCN^-) high absorbance in the far UV region or their potential to promote protein precipitate at pH 4.0.

However, sulphate and fluoride anions posed no such barrier and we were able to collect data from them. The effect of sodium fluoride (NaF) and sodium sulfate (Na_2SO_4) on the secondary structure of the protein was studied in detail. The changes in CD ellipticity at 222 nm for TgFNR at pH 4.0 and pH 7.5 in the presence of increasing concentrations of NaF or Na_2SO_4 are shown in Figs. 2A and B. At pH 7.5, there was no significant change in the secondary structure of TgFNR was observed in the presence of both salts, however a significant loss of CD ellipticity at 222nm was observed between 0-100 mM for NaF and 0–50 mM for Na_2SO_4 at pH 4.0. The increase in the salt concentrations beyond 50mM for Na_2SO_4 or 100 mM for NaF however resulted in the gain in secondary structure. The loss of secondary structure observed in NaF is due to the conversion of alpha-beta structure into the random coil, but in the case of Na_2SO_4, there was a total loss of CD signal (Fig. 2A & B inset). These observations suggest that the lower concentration of NaF or Na_2SO_4 causes significant loss in secondary structure at pH 4.0, however the structure is regained at higher salt concentrations. The loss of secondary structure observed at lower concentrations of kosmotropic salts such as NaF and Na_2SO_4 is highly unusual, because these salts are known to have a protein-stabilizing effect. [28]. The denaturing effect of kosmotropic ions on the structure of acidic pH stabilized conformation is quite rare and, to our knowledge, has never been observed with any other protein.

Salts have a significant impact on protein structure, solubility, and stability, and are known to do so in three ways: (i) by redistribution of charges close to the protein surface in all the salts, also known as the Debye-Huckel effect. (ii) by the Hofmeister effect, as caused by reorganization of water molecules around ions, i.e. by breaking or forming the water-structure. This effect is responsible for protein salting in or out

from aqueous solution. (iii) by a specific interaction between charged amino acid residues on the protein surface and salt ions. In our case, the specific interactions between salts (NaF and Na_2SO_4) and charged amino acids on the surface of protein could be a possible reason for the salt induced denaturation of TgFNR.

Under physiological settings, we had proposed that the open conformation of TgFNR is sustained by electrostatic repulsion between similar charged polar amino acids in the protein's core. The deionization of these amino acids occurs at acidic pH, resulting in the minimization/abolition of electrostatic interactions that exist under physiological conditions, resulting in a more compact conformation of TgFNR [15]. These charged amino acids may have undergone a structural rearrangement in acidic conditions, stabilizing the protein conformation by establishing specific interactions, specifically salt bridges on the protein surface. Furthermore, kosmotropic salts at lower concentrations may interact directly with these salt bridges, causing secondary structural loss. At higher salt concentration, the classical Hofmeister effect predominates where the denaturing effects of salts are reversed.

Salt dependent changes on thermal stability and structural cooperativity of TgFNR

The strong interactions exist between $NADP^+$- and FAD-binding domain of TgFNR resulting protein undergo cooperative unfolding during thermal denaturation. Lowering of the environmental pH causes selective modifications in the $NADP^+$- binding domain leading to the loss of interdomain interactions and non-cooperative unfolding [15]. To validate the destabilizing effect of both the salts on the stability and cooperativity of TgFNR, we measured changes in CD ellipticity at 222nm during thermal denaturation of TgFNR in the absence and presence of salts at pH 4.0 and 7.5. At pH 4.0, increasing the concentration of NaF leads to significant loss of secondary structure (up to 80%), but it improves TgFNR cooperativity (Fig. 3A). Furthermore, while the protein's noncooperative behaviour was preserved at low NaF concentrations, a considerable reduction of Tm (42.5^oC) was detected when compared to the conformation stabilized at pH 4.0. However, the thermal unfolding of TgFNR at higher concentrations of NaF was cooperative. In the case Na_2SO_4, even at its relatively low concentration, the cooperative unfolding of TgFNR was observed at pH 4.0. These observations suggest that in the presence of salts, the domain which is otherwise resistant to thermal denaturation at pH 4.0, becomes sensitive to it and the protein starts unfolding in a cooperative manner. However, Like NaF, the increasing concentration of Na_2SO_4 also destabilized the protein with decrease in Tm as compared to the conformation stabilized at pH 4.0 (Fig. 3B). As we know the proteins from thermophilic organisms have a higher number of surface salt bridges, which helps to stabilize them at higher temperatures. [29, 30]. It's possible that the relocalization of ionizable amino acids from the inside of proteins to the surface and their involvement in the development of surface salt bridges explains why acidic pH improves TgFNR's thermal stability. By interfering with these salt bridges, kosmotopic salts may be disrupting the acidic pH-induced conformation of TgFNR. We further studied the effect of NaF and Na_2SO_4 on thermal denaturation of TgFNR at pH 7.5 (Fig. 3C & D). At low salts concentration (upto 100 mM) a considerable decrease in the Tm of TgFNR was observed but the higher

concentrations (up to 500 mM) result in an increase in its Tm. TgFNR's thermal denaturation was irreversible at pH 7.5 under most of the experimental conditions we tested, since the protein aggregated, making it impossible to calculate the thermodynamic parameters.

Urea induced equilibrium unfolding of TgFNR in the absence and presence of salts at neutral pH

Salt dependent investigations were also carried out as a complement to thermal denaturation by measuring the CD ellipticity at 222 nm as a function of urea concentration at pH 7.5. Sodium chloride was also utilized in this experiment since it is a neutral salt that falls between kosmotropic and chaotropic salts in the Hofmeister series. The urea induced unfolding of TgFNR in the absence and presence of NaCl, NaF, and Na_2SO_4 are shown in Figs. 4A, B, and C, respectively. All the curves are indicative of a cooperative transition from the native to unfolded states. At pH 7.5, increasing the concentration of NaCl, correspondingly lowers the Cm value for TgFNR denaturation, but a biphasic impact of Na_2SO_4 and NaF for such denaturation was seen, with the Cm value decreasing and then increasing. Lower salt concentrations caused significant protein instability, but this effect was reversed at higher salt concentrations. Because of the salt's limited solubility in aqueous solvent, the effect of higher NaF concentrations (> 250 mM) could not be investigated. To calculate the thermodynamic parameters from this data, individual denaturation curves were fitted according to a two-state model yielding the free energy of unfolding extrapolated to zero denaturant concentration, ΔG_U^{H2O} and the m value [31]. The obtained results were plotted as function of varying concentration of Na_2SO_4, NaF and NaCl (Fig. 5A-F). A biphasic effect of kosmotropic salts (Na_2SO_4 and NaF) was observed on the thermodynamic stability of TgFNR at neutral pH. Evaluation of ΔG_U^{H2O} indicates that all three salts (Na_2SO_4, NaF and NaCl) significantly reduced the thermodynamic stability of TgFNR at lower concentrations. However, a concomitant recovery in free energy was observed with increasing concentration of kosmotropic salts but not with sodium chloride. In the presence of various salts, the m values for TgFNR unfolding exhibit no consistent dependency on the salt concentrations (data not shown). Moreover, a marked decrease in Cm values observed at lower concentrations of salts indicating the that the protein was destabilized under all of these conditions. The destabilization effect is gradually reversed with the increasing concentrations of kosmotropic salts but the similar reversal in Cm value was not observed for sodium chloride. It is unusual for these salts to have such an effect on protein stability, and it has only been reported for prion protein so far. The function of salt bridges and long-range electrostatic contact in the destabilizing effect of hofmeister series salts has only been investigated once, for prion protein [12], where the function of salt bridges and long-range electrostatic contact in the destabilizing effect of hofmeister series salts has been explored. Similarly, salt bridges have been implicated in the destabilization of methionine amino peptidase from the hyperthermophile *Pyrococcus furiosus* by KCl. [32]. Despite the fact that the high-resolution structure of TgFNR has not yet been published, the primary amino acid sequence of TgFNR contains a large number of charged amino acid residues (24 Arginine 6.76%; 20 Lysine- 5.63%; 11 Histidine 3.1%; 15 Glutamic acid 4.23% and 23 Aspartic acid 6.48%). We speculate that some of these charged amino acids might contribute to the stabilization of native structure of TgFNR through formation

of salt bridges and affected by the presence of salts. However, the real estimate of salt bridges can only be obtained once high-resolution structure of TgFNR is known.

Conclusions

The current study provides deeper insights on the role of electrostatic interactions in the stabilization of TgFNR's natural conformation. The low concentrations of kosmotropic salts, which are known to stabilize proteins are found to significantly perturbed the secondary structure of TgFNR stabilized at pH 4.0. This is quite unique and, to our knowledge, has never been reported for any other protein. It's also worth noting that sulphate and fluoride have biphasic effects on the structure and stability of TgFNR. This biphasic response shows that salts saturate the structure of TgFNR at relatively low concentrations, while the classical kosmotropic effect dominates at higher concentrations, resulting in gradual stabilization as expected by the Hofmeister series. The unique destabilizing effect of salts on the structure and stability of TgFNR suggests that salt bridges play a role in the stabilization of TgFNR's native conformation.

Declarations

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Conflicts of interest

The authors declare no conflict of interest.

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Figures

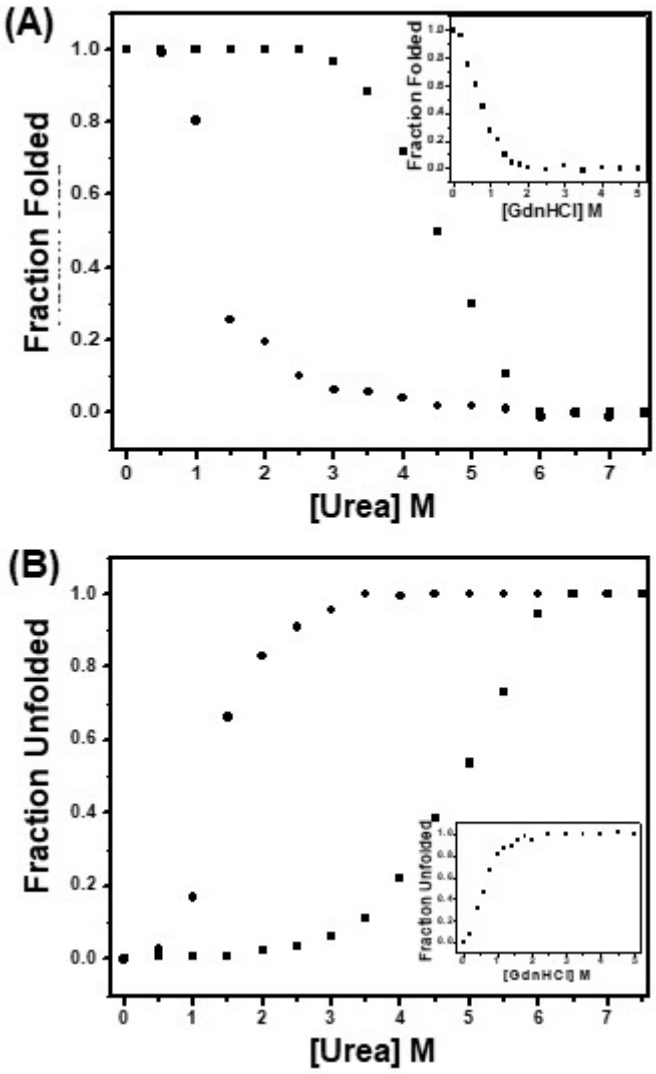

Figure 1

The unfolding of TgFNR. Effect of increasing urea concentration on the CD ellipticity at 222 nm (Panel A) and changes in the FAD fluorescence intensity (Panel B) of TgFNR at pH 7.5 (solid squares) and 4.0 (solid circles). GdnHCl induced changes in CD ellipticity at 222 nm (Panel A inset) and FAD fluorescence intensity (Panel B inset) at pH 4.0. A linear extrapolation of the baseline in the pre- and post-transional regions used for the fraction of folded/unfolded enzyme within the transition region by assuming the two-state mechanism of unfolding.

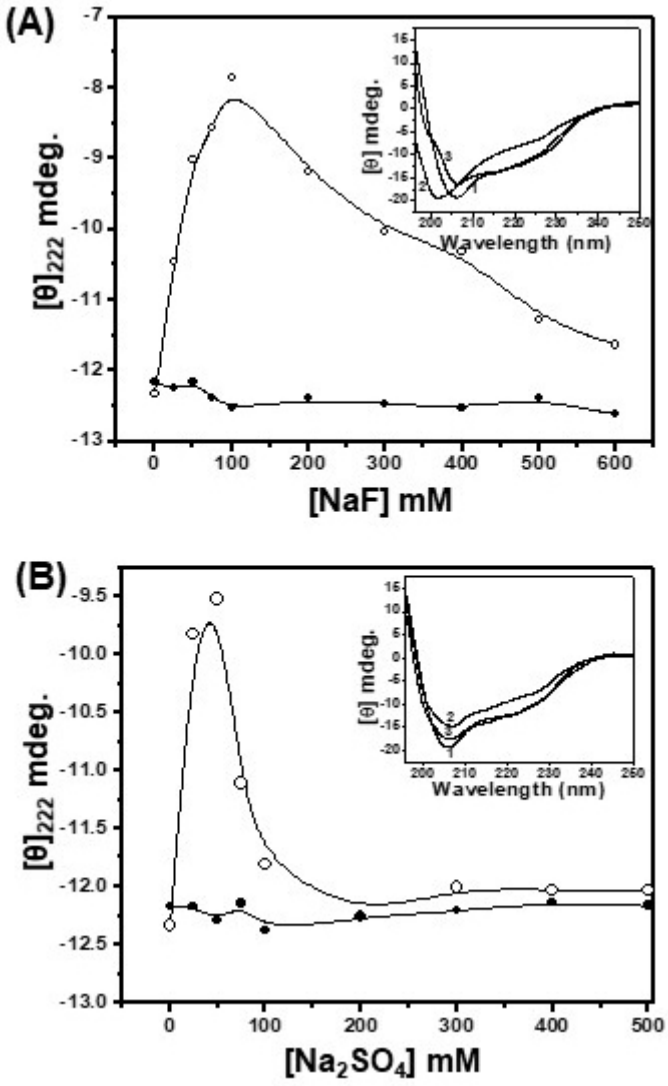

Figure 2

Effect of salts on secondary structure of TgFNR. Changes in the CD ellipticity at 222 nm for TgFNR in the presence of sodium fluoride (Panel A) and sodium sufate (Panel B) at pH 7.5 (solid circle) and pH 4 (open circle) respectively. Inset of Panel A show the CD spectra of TgFNR in the presence of 0 (profile 1), 100 mM (profile 2) and 600 mM (profile 3) sodium fluoride at pH 4.0. Inset of Panel B show the CD spectra of TgFNR in presence of 0 (profile 1), 50mM (profile 2) and 500 mM (profile 3) of sodium sulphate at pH 4.0.

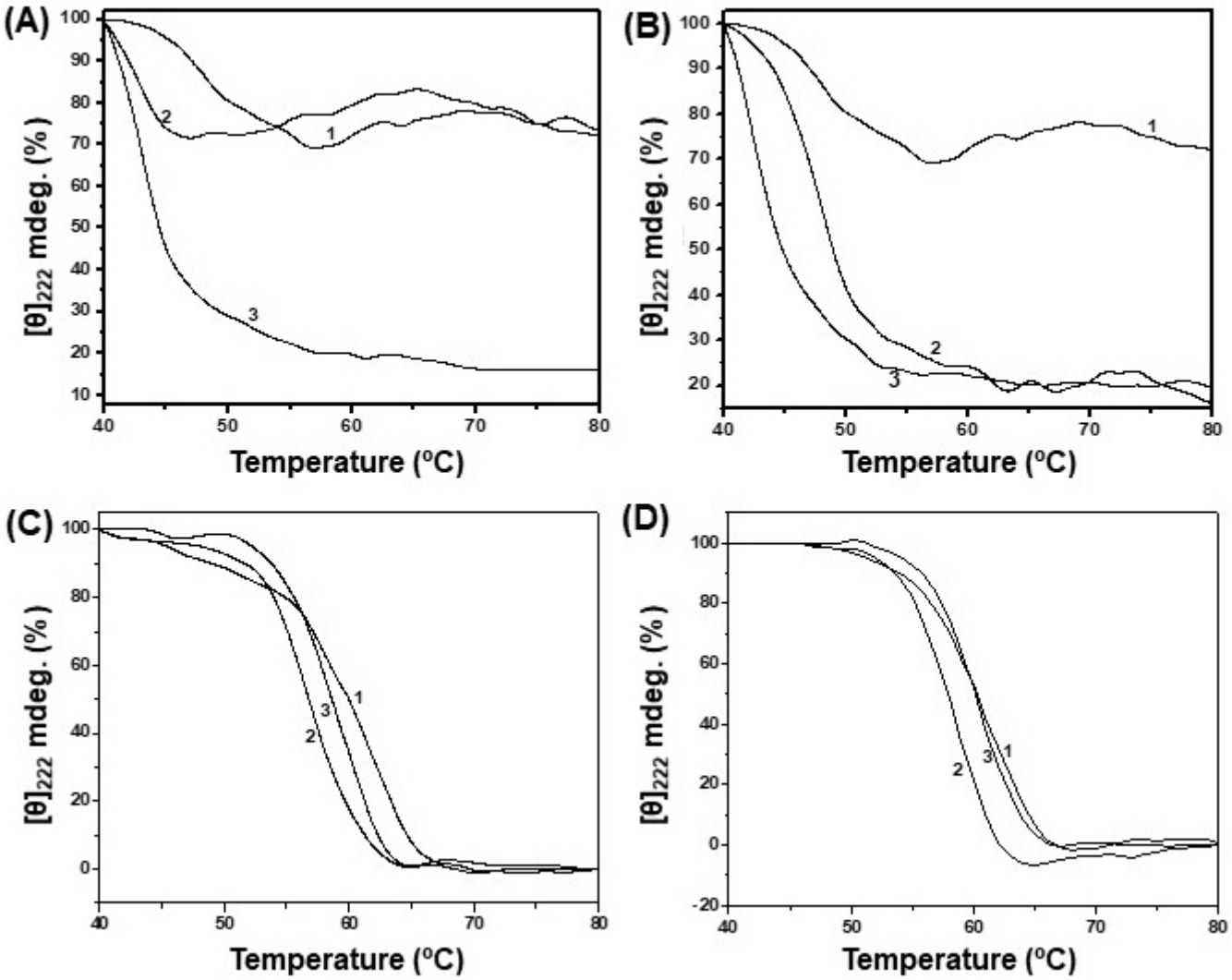

Figure 3

Effect of salts on thermal denaturation of TgFNR. Thermal denaturation of TgFNR at pH 4.0 (Panel A & B) and pH 7.5 (Panel C & D) with increasing concentrations of sodium fluoride (Panel A & C) and sodium sulfate (Panel B & D) as monitored by loss of CD signal at 222nm with increasing temperature. In panel A, the profiles 1 to 3 represent protein samples in absence and presence of 50 and 500 mM of NaF respectively. In panel B, the profiles 1 to 3 represent protein samples in absence and presence of 50 and 500 mM of Na_2SO_4 respectively. In Panel C, the profiles 1 to 3 represent protein samples in absence and presence of 100 and 250 mM NaF, respectively. In Panel D, the profiles 1 to 3 represent protein samples in absence and presence of 100 and 500 mM of Na_2SO_4 respectively. The data has been represented as percentage with the value observed for protein at 40°C taken as 100 percent.

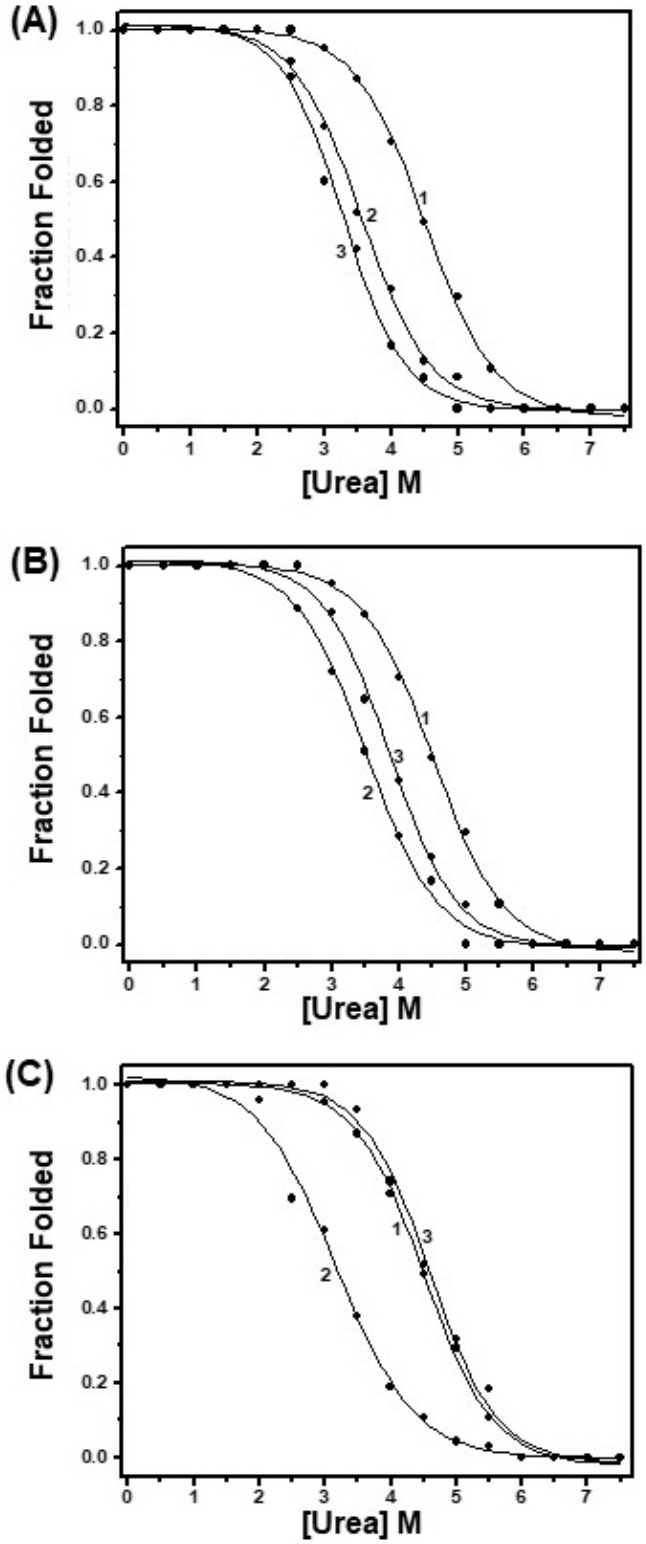

Figure 4

The urea induced unfolding (CD ellipticity at 222nm) of TgFNR at increasing concentrations of sodium chloride (Panel A), sodium fluoride (Panel B) and sodium sulfate (Panel C). In Panel A and C, profiles 1 to 3 represent protein samples at zero, 100mM and 500mM NaCl and Na_2SO_4 respectively. In Panel B, the profiles 1 to 3 represent protein samples at zero, 100mM and 250 mM of NaF. For all the curves a linear

extrapolation of the baseline in the pre and post transitional regions used to calculate the fraction of folded enzyme within the transition region by assuming the two-state mechanism of unfolding.

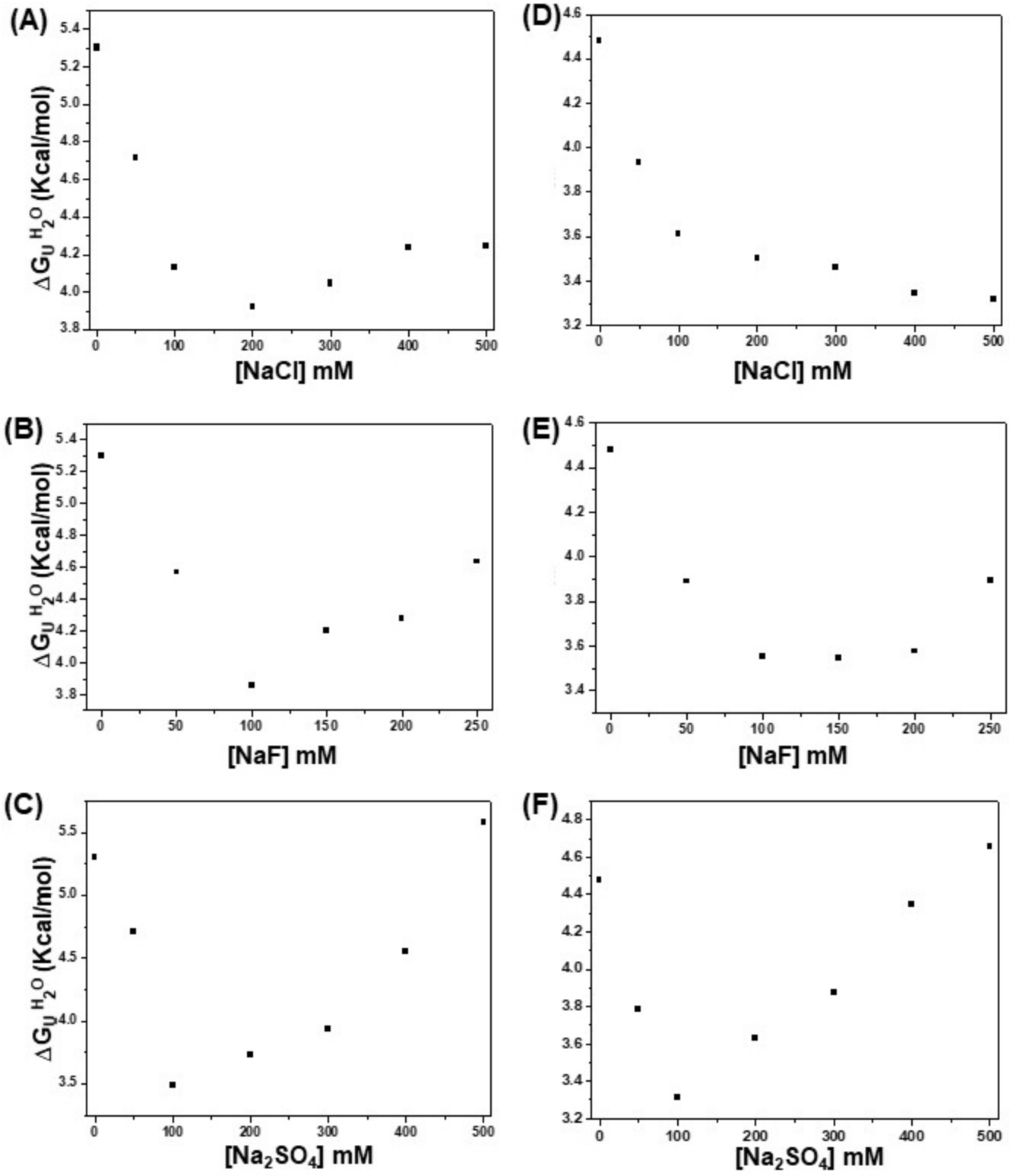

Figure 5

The effect of increasing concentrations of different salts (sodium chloride, sodium fluoride and sodium sulphate on DG_U^{H2O} (Panel A, B, C) and Cm value (Panel D, E, F) of TgFNR at pH 7.0 and at 25°C.