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Guanidine Hydrochloride- and Urea-Induced Unfolding of *Toxoplasma gondii* Ferredoxin-NADP$^+$ Reductase: Stabilization of a Functionally Inactive Holo-Intermediate

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Usually during the folding/unfolding of flavoproteins, an apo-intermediate is stabilized before global unfolding of the enzymes occurs. However, stabilization of a holo-intermediate has also been reported for a few flavoproteins. We have studied the unfolding of *Toxoplasma gondii* ferredoxin-NADP$^+$ reductase (TgFNR) using GdnHCl and urea. A functionally inactive holo-intermediate of the enzyme was found to be stabilized during this unfolding process. The intermediate species had cofactor FAD bound to it, but it showed free movement due to which the stabilized intermediates were functionally inactive. The native TgFNR behaves cooperatively with the two structural domains interacting strongly with each other. The denaturants GdnHCl and urea, at low concentrations, were found to interact selectively with the NADP$^+$-binding domain of TgFNR and to induce structural modifications in it. These selective modifications in the protein molecule lead to loss of interactions between two domains and the enzyme behaved non-cooperatively resulting in stabilization of an intermediate species. Significant differences in the structural properties of the GdnHCl- and urea-stabilized holo-intermediates of TgFNR were observed. Comparison of the unfolding pathway of TgFNR (a plant-type FNR) with that of FprA (a GR-type FNR) demonstrates that they follow very different pathways of unfolding.

Key words: flavin adenine dinucleotide, flavoproteins, folding/unfolding, holo-intermediate, protein stabilization/destabilization.

Abbreviations: AdR, adrenodoxin reductase; ANS, 1-anilino-8-naphthalene sulfonate; CD, circular dichroism; Cm, midpoint of chemical denaturation; FAD, flavin adenine dinucleotide; FNR, ferredoxin-NADP$^+$ reductase; FprA, *Mycobacterium tuberculosis* NADPH-ferredoxin reductase; GdnHCl, guanidine hydrochloride; GR, glutathione reductase; ONFR, oxygenase-coupled NADH-ferredoxin reductase; SEC, size exclusion chromatography; TgFNR, *Toxoplasma gondii* ferredoxin-NADP$^+$ reductase; T_m, midpoint of thermal denaturation.

Folding/unfolding and stability studies have largely been carried out for small monomeric proteins or domains of multidomain proteins, because they display simple two-state cooperative unfolding. However, such studies with larger multidomain or multimeric proteins are complex and often involve stabilization of intermediates (*1*–*3*). Characterization of such intermediate states is central to understand the process of protein folding and stability as they provide useful insights in the forces that stabilize the partially folded intermediates (*4*, *5*). Furthermore, these intermediate states are also involved in several physiological and pathological processes inside the cell (*6*, *7*). Evolution of multidomain proteins may be considered as most significant acquisition as they involve synchronous folding at multiple sites along the nascent polypeptide chain and specific interactions between these domains lead to generation of functional three dimensional structures of proteins (*8*). In the case of many multidomain proteins, the active site of enzymes is often positioned between the domains (*9*). To further extend studies on multidomain proteins, ferredoxin-NADP$^+$ reductase (FNR) seems to be an ideal candidate.

FNR is a flavoprotein that catalyses reversible electron transfer between two molecules of the obligatory one electron carrier ferredoxin (Fd) and a single molecule of NADP(H) that is consequently used in Calvin cycle for CO_2 fixation in the case of chloroplast of higher plant or algae and cyanobacteria (*10*, *11*). The existence of such redox system has also been reported in apicomplexan parasites, where it is assumed that the reduced ferredoxin provides reducing power to various ferredoxin dependent biosynthetic pathways (*12*, *13*). Recently, it has been shown that a reconstituted *in vitro* electron transport system based on recombinant *Plasmodium falciparum* FNR and Fd is effective in providing electrons to (E)-4-hydroxy-3-methyl-but-2-enyl-diphosphate reductase (Lyt B) (*14*). This enzyme

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synthesizes activated isoprene units as the last step of the non-mevalonate isoprenoids biosynthesis, an apicoplast localized pathway known to be essential for the parasite (*15*).

FNRs have been grouped into two phylogenetic/structural families referred to as plant-type and glutathione reductase (GR)-type. The plant-type FNRs comprise the enzymes from plastid and bacterial sources whereas the GR-type enzymes comprise those of AdR-like and ONFR-like (*16*). Apicomplexan FNRs, like *Toxoplasma gondii* ferredoxin-$NADP^+$ reductase (TgFNR) belong to the plant-type FNR family of enzymes, for which detailed functional and structural characterization have been summarized in recent reviews (*10*, *17*, *18*). The FprA is a GR-type FNR. Structurally, both types of FNRs possess two-domain organization, and the active site is located at the interface between the FAD- and $NADP^+$-binding domains. The $NADP^+$-binding domain of both the FNR families have similar structures however, the FAD-binding domain shows distinct differences. In plant-type FNRs, the FAD-binding domain is formed by the N-terminal portion of polypeptide chain, whereas, in GR-type FNRs, two discontinuous segments of polypeptide form the FAD-binding domain (*16*). We had earlier reported the unfolding pathway of a GR-type FNR the *Mycobacterium tuberculosis* FNR, FprA, for which a multistep unfolding with stabilization of an apo-intermediate was reported (*19*).

While the crystal structure of *Plasmodium falciparum* FNR has recently been reported, the three-dimensional structure of TgFNR is still lacking however, its structure has been modeled on the basis of the 3D structure of non photosynthetic maize root FNR (*20–22*). According to the predicted structure, TgFNR is made up of two structural domains, the $NADP^+$- and FAD-binding domains, and the FAD cofactor is sandwiched in the large cleft between the two domains. To get insight into the unfolding pathway of the plant-type FNR, we thought it appropriate to study the unfolding of TgFNR. In the present investigation, we have characterized the structural and functional changes associated with GdnHCl- and urea-induced unfolding of TgFNR. Biophysical techniques including fluorescence spectroscopy, circular dichroism in combination with size exclusion chromatography (SEC), limited proteolysis, ANS binding and activity assay have been used in the study.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used in the study were purchased from Sigma-Aldrich Chemical Company, St Louis, USA, and were of highest purity available. All chromatographic columns were purchased from GE Healthcare Biosciences, with the exception of Ni–NTA agarose, which was from Qiagen.

Overproduction and Purification of TgFNR—The overproduction and purification of recombinant TgFNR was carried out as described earlier (*23*). The purified proteins showed > 95% purity, as quantitated from ESI–MS and SDS–PAGE analysis (data not shown).

Enzymatic Activity Assay—Enzyme catalysed reactions were monitored continuously using a UV1650 PC visible spectrophotometer (Simadzu). Diaphorase activity was measured in 100 mM Tris–HCl (pH 7.5) containing 1 mM DTT at 25°C with $K_3Fe(CN)_6$ as electron acceptor and NADPH as reductant (*13*). The enzyme was incubated in the absence and presence of increasing concentrations of GdnHCl and urea for 6 h, and the activity was measured by monitoring the decrease in absorbance at 420 nm. The obtained values were normalized by subtracting the baseline recorded for the buffer alone under similar conditions.

Fluorescence Spectroscopy—Fluorescence spectra were recorded with Perkin–Elmer LS 50B spectroluminescencemeter in a 5-mm path length quartz cell. Protein concentration was 3.0 μM for all experiments, and the measurements were carried out at 25°C. For monitoring tryptophan and FAD fluorescence, the excitation wavelengths of 285 and 445 nm, respectively, were used, and the spectra were recorded between 300–400 nm and 450–550 nm, respectively. The FAD fluorescence polarization was measured at a fixed wavelength of 525 nm. For ANS-binding experiments, aliquots of protein (5 μM) were incubated in the absence and presence of increasing concentrations of GdnHCl and urea for 6 h at 25°C. ANS was added to a final concentration of 50 μM in these solutions and incubated further for 1 h at 25°C. The fluorescence emission spectra were recorded with excitation wavelength set at 365 nm, and recorded spectra were corrected for ANS fluorescence obtained in buffer alone.

Circular Dichroism Measurements—CD measurements were made with a Jasco J810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The CD spectra were recorded at enzyme concentration of 3.0 μM in a cell of 0.5-mm path length at 25°C. The obtained values were normalized by subtracting the baseline recorded for the buffer alone under similar conditions.

Thermal Denaturation—Thermal denaturation of TgFNR (3.0 μM for far-UV CD and 20 μM for near-UV CD) at desired denaturant concentrations were studied by monitoring changes in CD ellipticity at 222 nm or 375 nm as a function of temperature on a Jasco J810 spectropolarimeter equipped with peltier temperature controller system. The measurements were carried out in 10 mM Tris–HCl (pH 7.5) containing 1 mM DTT and desired denaturant concentrations. The samples were equilibrated at the starting temperature by incubating for half an hour before the measurements were made. Samples were heated at a constant rate of 1°C/min in a cell with 1-mm path length.

SEC—The SEC experiments were carried out on the Superdex 75 HR 10/300 column (manufacturer exclusion limit 75 kDa) interfaced with AKTA fast performance liquid chromatography (GE Healthcare Biosciences). The column was equilibrated and run with 10 mM Tris–HCl (pH 7.5) containing 1 mM DTT and desired denaturant concentrations at 25°C. Two hundred microlitres of the sample were injected in the column and run at 25°C at a flow rate of 0.3 ml/min, with detection at 280 and 445 nm.

Limited Proteolysis—TgFNR (1 mg/ml) in 50 mM Tris–HCl (pH 7.5) containing 1 mM DTT was incubated in the absence and presence of desired concentrations of GdnHCl and urea for 6 h at 25°C followed by incubation with trypsin (protease: protein w/w ratio of 1:50) for 1 h at 25°C. The reactions were stopped by addition of 1 mM PMSF followed by precipitation with 5% TCA. The resulting precipitate was washed with chilled acetone twice, and the pellet obtained was dissolved in 50 mM Tris–HCl (pH 7.5) containing 1 mM DTT and analysed on 12% SDS–PAGE.

RESULTS

We have studied the effect of GdnHCl- and urea-induced changes in the structural and functional properties of TgFNR. Time-dependent changes in the structural parameters and enzymatic activity of TgFNR at increasing GdnHCl or urea concentrations (0.5, 2 or 6 M) were monitored to standardize the incubation time required to achieve equilibrium under these conditions. Under all the conditions studied, the changes occurred within maximum of about 6 h with no further alterations in the values obtained up to 24 h (data not shown). These observations suggest that a minimum time of about 6 h is sufficient for achieving equilibrium under any of the denaturing conditions studied.

GdnHCl-Induced Changes in Structural and Functional Properties of TgFNR—TgFNR contains a single tightly but non-covalently bound FAD molecule and four tryptophan residues. In the native conformation, the FAD moiety and tryptophan residues are buried inside the protein core (*24*). Figure 1A summarizes the changes in FAD fluorescence intensity and tryptophan emission maxima of TgFNR with increasing concentrations of GdnHCl. A sigmoidal dependence of FAD fluorescence intensity with maximum enhancement of about 20 times and a significant red shift in the emission λ_{max} from 339 to about 348 nm was observed with increasing GdnHCl concentrations between 0.75 and 1.75 M. For TgFNR, such an enhancement in FAD fluorescence intensity is associated with release of protein-bound FAD molecule (*25*, *26*). As the exposed tryptophan residues in an unfolded protein show emission maxima between 348 and 356 nm (*27*), these observations suggest that treatment of TgFNR with higher concentrations of GdnHCl results in dissociation of FAD cofactor from the enzyme along with solvent exposure of buried tryptophan moieties due to unfolding of TgFNR.

The interesting observation is that the GdnHCl-induced unfolding of TgFNR as observed from changes in FAD and tryptophan fluorescence shows a two-state protein unfolding pathway with similar Cm value of about 1.2 M GdnHCl (Fig. 1A). To test whether this two-state unfolding of TgFNR reflects the disruption of overall structure of the protein or is indicative of only local unfolding, we studied the GdnHCl-induced alterations in the secondary structure of protein. In the far-UV region, the CD spectrum of native TgFNR shows the presence of substantial α-helical conformation (*24*) (Fig. 1B inset). Figure 1B summarizes the effect of increasing concentrations of GdnHCl on the CD ellipticity at 222 nm for TgFNR. Between 0.0 and 0.5 M GdnHCl, an enhancement in CD ellipticity for native TgFNR was observed. However, further increase in GdnHCl concentrations between 0.5 and 1.75 M GdnHCl resulted in a large gradual decrease in CD ellipticity, and almost complete loss of CD signals was observed at about 2 M GdnHCl. The Cm value of this main transition corresponds to 1.2 M GdnHCl, which was similar to that observed from changes in tryptophan and FAD fluorescence mentioned earlier. In order to confirm that the enhancement in CD signals at low-GdnHCl concentrations are due to GdnHCl-induced stabilization of secondary structure of TgFNR and not because of salt effects, we carried out similar studies in the presence of NaCl. No significant change in CD signals of native TgFNR were observed up to 1 M NaCl (Fig. 1B). These observations demonstrate that the backbone or secondary structure of TgFNR is apparently stabilized on treatment of enzyme with low concentrations of GdnHCl. A similar stabilization of secondary structure of proteins at low-GdnHCl concentrations had been reported for several proteins (*28*, *29*). At high GdnHCl concentrations, complete unfolding of the enzyme was observed. The above results provided a possibility of stabilization of a partially unfolded intermediate of TgFNR at low-GdnHCl concentrations.

The effect of GdnHCl on the enzyme activity of TgFNR was investigated by monitoring the decrease in absorbance at 420 nm in standard reaction mixture detailed in EXPERIMENTAL PROCEDURE section, and results are summarized in Fig. 1C. An exponential decrease in enzymatic activity from 100% to about 10% was observed between 0.0 and 0.5 M GdnHCl concentrations and above 1 M GdnHCl, no enzymatic activity was seen. A strong correlation between alterations in enzymatic activity and FAD fluorescence polarization has been reported to exist in TgFNR (*24*). Hence, we monitored the changes in FAD fluorescence polarization of TgFNR at increasing GdnHCl concentrations (Fig. 1D). A sharp decrease in fluorescence polarization with increasing GdnHCl concentrations was observed between 0.0 and 1 M GdnHCl and above this denaturant concentration a complete loss of polarization occurs. These results along with that of enzymatic activity support the earlier suggestion that slight variations in orientation of the protein bound FAD lead to complete loss of enzymatic activity of TgFNR (*24*).

The results of GdnHCl-induced structural and functional changes in the enzyme demonstrate that the stabilization of an enzymatically inactive partially unfolded intermediate of TgFNR at low GdnHCl concentration. This is also supported by the observation of ANS binding to the GdnHCl-stabilized intermediate of the enzyme. ANS is a hydrophobic dye that binds to hydrophobic clusters present in the proteins (*30*). Figure 1E summarizes the changes in the fluorescence intensity of ANS on incubation of TgFNR with increasing concentrations of GdnHCl. A single peak centered at about 0.5 M was observed between 0.0 and 1 M GdnHCl suggesting the presence of exposed hydrophobic clusters in the partially unfolded intermediate of TgFNR stabilized at about 0.5 M GdnHCl. The refolding efficiency of partially folded intermediate (obtained at 0.5 M GdnHCl)

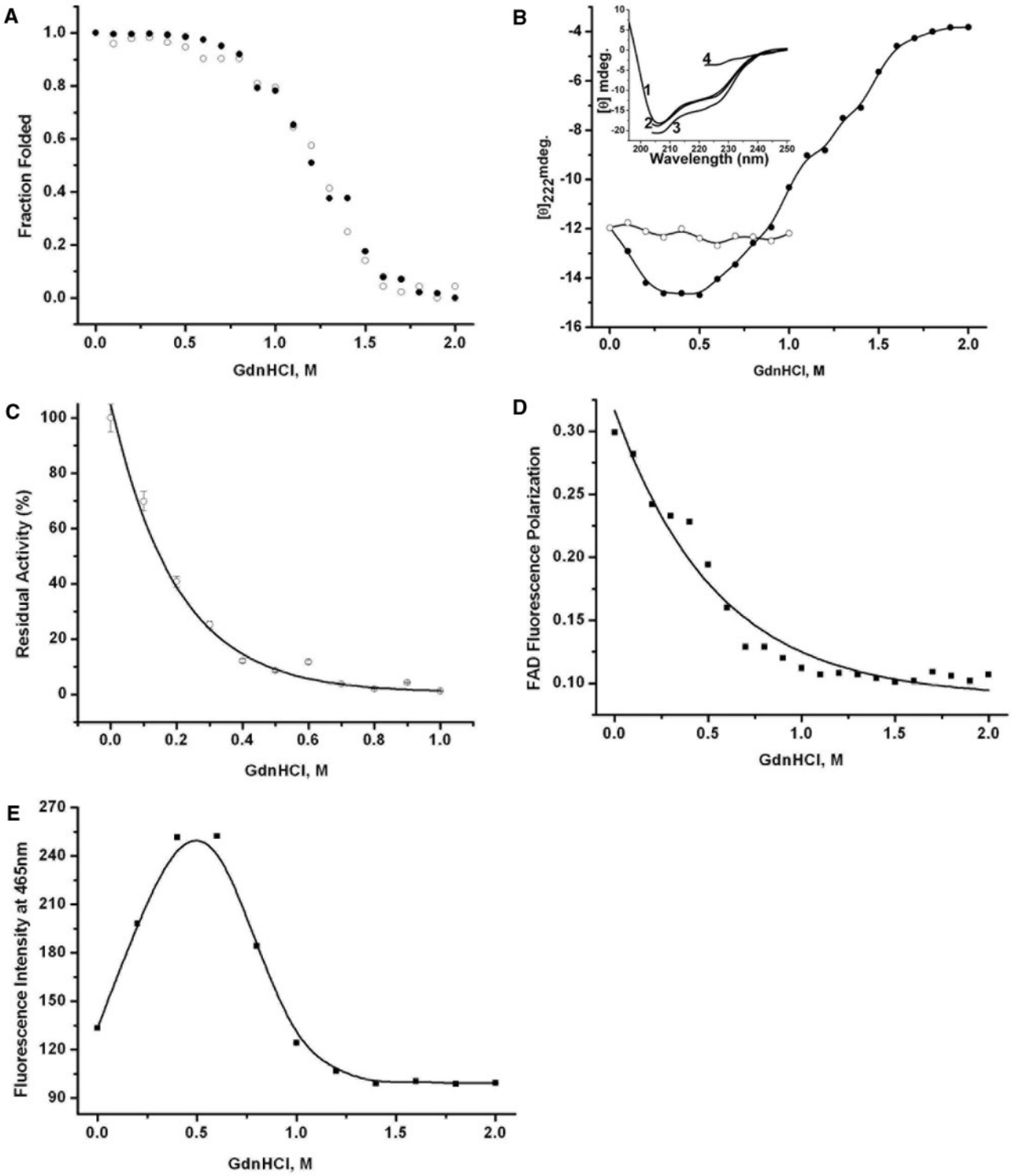

Fig. 1. **Changes in molecular properties of TgFNR in the presence of increasing concentrations of GdnHCl at pH 7.5 and 25°C.** (A) GdnHCl induced unfolding transitions of TgFNR as obtained from changes in FAD fluorescence intensity (solid circle) and tryptophan emission maxima (open circle) at increasing GdnHCl concentrations. A linear extrapolation of the baselines in the pre- and post-transitional regions was used to determine the fraction of the folded enzyme within the transition region by assuming a two-state mechanism of unfolding. (B) GdnHCl (solid circle) and NaCl (open circle) induced changes in the secondary structure of TgFNR as followed by monitoring changes in CD ellipticity at 222 nm of TgFNR at increasing concentrations of GdnHCl. The inset shows far-UV CD profiles of native TgFNR (profile 1) and those in the presence of 1 M NaCl (profile 2), 0.5 M (profile 3) and 2.0 M GdnHCl (profile 4). (C) Changes in the enzymatic activity of the TgFNR on treatment with increasing concentrations of GdnHCl. The data are expressed as relative activity taking the activity of native enzyme as 100%. The data represent mean ± SD of three independent measurements. (D) Changes in FAD fluorescence polarization. (E) Changes in fluorescence intensity of ANS on incubation with TgFNR, treated with increasing concentrations of GdnHCl.

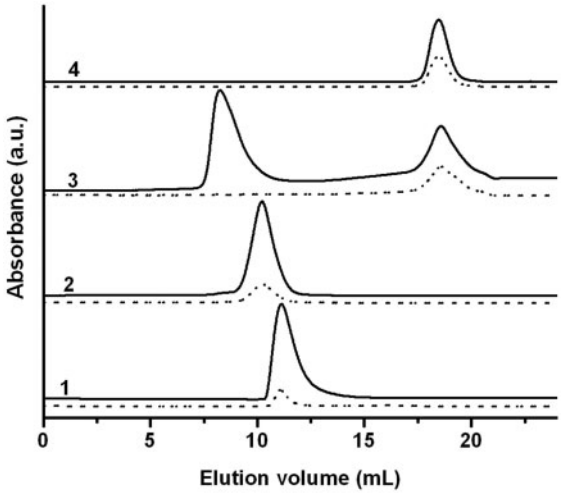

Fig. 2. **GdnHCl induced changes in the molecular dimension of TgFNR at pH 7.5 and 25°C.** SEC profiles on superdex 75HR column, profiles 1–4 represent samples of native, 0.5 M, 2 M GdnHCl-treated TgFNR and FAD alone in the presence of 2 M GdnHCl, respectively. Solid and dotted lines represent absorbance at 280 nm and at 445 nm, respectively.

and unfolded TgFNR (obtained at 6 M GdnHCl) was found to be 100% and about 67%, respectively (data not shown).

Characterization of GdnHCl Stabilized Unfolding Intermediate of TgFNR—For studying the effect of GdnHCl, on the molecular dimension of TgFNR, SEC studies on superdex 75 HR 10/30 column (manufacturer exclusion limit 75 kDa) were carried out. The signals were detected at 280 and 445 nm for monitoring change in microenvironment of tryptophan residues and FAD cofactor, respectively (Fig. 2). Under native conditions, TgFNR is stabilized in a relatively open conformation (*23*, *24*). In the presence of 0.5 M GdnHCl, the elution peak showed a slight shift to lower retention volume of 10.20 ml indicating slight opening of the native conformation of the enzyme. However, the FAD moiety was found to be associated with the enzyme under these conditions as the signals at 280 and 445 nm were found to be overlapping. For 2 M GdnHCl-treated TgFNR, an elution volume of about 8.0 ml was observed, which corresponds to the void volume of the column. Such large increase of hydrodynamic radii was expected as the enzyme was completely unfolded under these conditions. Furthermore, the FAD moiety was found to be dissociated from the enzyme as the signal at 445 nm was shifted to significantly high-elution volume (Fig. 2).

The GdnHCl-Stabilized Intermediate of TgFNR Shows Non-Cooperative Behaviour—The FNR flavoproteins are made up of two structural domains, the NADP⁺- and FAD-binding domains. In the native TgFNR, the two structural domains interact strongly as a result of which a sigmoidal transition with complete loss of secondary structure was observed during thermal unfolding of the enzyme. Figure 3A shows the thermal denaturation profile of TgFNR at different concentrations of GdnHCl as monitored by loss of the CD signals at 222 nm with

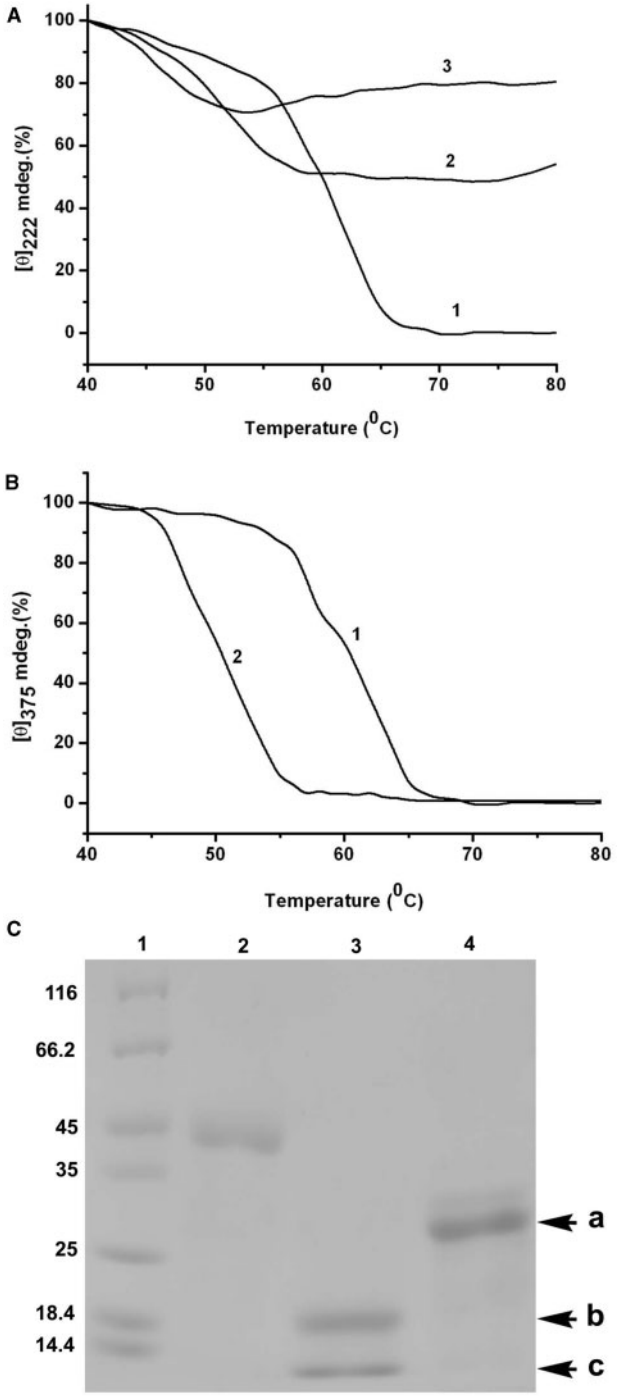

Fig. 3. **(A) Thermal unfolding of TgFNR as studied by monitoring the loss of CD signals at 222 nm with increasing temperature.** The curves 1–3 represent sample of native, 0.5 M and 1 M GdnHCl-treated TgFNR, respectively. (B) Thermal unfolding of TgFNR as studied by monitoring the loss of CD signals at 375 nm (corresponding to FAD moiety) with increasing temperature. The curves 1 and 2 represent protein samples at 0.0 and 0.5 M GdnHCl, respectively. For both the panels A and B, the data has been represented as percentage with the value observed for protein at 40°C taken as 100%. (C) SDS–PAGE profile of digestion of GdnHCl stabilized intermediate, lanes 1–4 represent molecular weight markers, purified TgFNR, TgFNR in the absence and presence of 0.5 M GdnHCl after treatment with trypsin, respectively.

increasing temperature. In the presence of GdnHCl concentrations of 0.5 and 1.0 M, a single sigmoidal reversible transition but with only about 50% and 21% loss of CD signals, respectively, were observed however, the thermal unfolding of native enzyme was irreversible as precipitation of solution was observed at the end of scan. These results indicate that at low-GdnHCl concentrations, one of the domains of TgFNR undergoes structural modifications, which results in its stabilization against thermal denaturation. The gradual loss of interactions between two structural domains under these conditions is also supported by the observations that significant lowering of the Tms at 0.5 M and 1.0 M GdnHCl as compared with the untreated protein was observed. To characterize the structural domain of TgFNR that undergoes destabilization on treatment with GdnHCl, near UV CD studies were carried out. Figure 3B shows the loss of CD signals at 375 nm of TgFNR with increasing temperature at 0.0 and 0.5 M GdnHCl. Sigmoidal transitions with almost complete loss of CD signals were observed for both the samples. Furthermore, T_m values of about 60°C and 50°C were found to be associated with the transitions at 0.0 and 0.5 M GdnHCl-treated samples, respectively. These Tms are close to those observed for the partial loss of secondary structure of TgFNR under similar conditions (Fig. 3A). This suggests that the partial unfolding of TgFNR at 0.5 M GdnHCl corresponds to unfolding of FAD-binding domain only implying that $NADP^+$-binding domain undergoes structural modifications on treatment with GdnHCl and becomes resistant to thermal denaturation.

Conformational Flexibility of GdnHCl Stabilized Intermediate of TgFNR—Limited proteolytic digestion has been used extensively to investigate the conformational flexibility of proteins, because it is governed by stereochemistry and accessibility of the protein substrate as well as proteolytic enzymes (*31*, *32*). TgFNR possesses several lysine and arginine residues scattered all over the primary amino acid sequence. So, trypsin seems to be an appropriate choice of protease to study the conformational differences that possibly exist between native and GdnHCl-stabilized intermediate of TgFNR. The proteolytic susceptibility of TgFNR to trypsin under native conformation as well as that in the presence of 0.5 M GdnHCl was analysed. Figure 3C shows the SDS–PAGE profile of TgFNR incubated in the absence and presence of 0.5 M GdnHCl with trypsin at 25°C. To map the trypsin cleavage sites in TgFNR, in the absence and presence of GdnHCl, the major protein fragments obtained on SDS–PAGE were subjected to N-terminal sequencing and molecular mass determinations by ESI–MS, and the results are summarized in Table 1. In all the cases, we could not obtain any fragment corresponding to N-terminal (1–96 residues) part, i.e. FAD-binding domain of TgFNR, suggesting that this part of enzyme is flexible and hence highly vulnerable to proteolysis by protease. For native TgFNR, two major proteolytic fragments of molecular mass of about 19.7 and 8.89 kDa corresponding to the protein fragment N^{97}-K^{279} and N^{280}-Y^{355} were obtained. However, for TgFNR treated with 0.5 M GdnHCl, a major protein band of molecular weight 26.56 kDa corresponding to protein fragment from R^{118}-Y^{355} was obtained. This suggests that TgFNR intermediate stabilized at 0.5 M GdnHCl has conformation where the K^{279}-N^{280} clevage site, which is exposed in the native conformation of the enzyme, is not accessible to the protease. As the K^{279} residue is present in the $NADP^+$-binding domain of the enzyme hence, the above observation indicates that this domain undergoes structural modifications at low GdnHCl concentrations.

Table 1. **Identification of the proteolytic fragments obtained by limited proteolysis of TgFNR.**

Bands on SDS–PAGE	Molecular mass (kDa)	N-terminal sequence	Putative fragment
a	26.56	RLLPRIY	Arg118-Tyr355
b	19.7	NSVQPD	Asn97-Lys279
c	8.89	NPQGKK	Asn280-Tyr355
d	28.6	NSVQPD	Asn97-Tyr355

Changes in the Molecular Properties of TgFNR Associated with Urea-Induced Unfolding—Although urea and GdnHCl are believed to have similar mode of action, GdnHCl has ionic and chaotropic effects, whereas urea has only chaotropic effects (*33*, *34*).

Figure 4A summarizes the urea-induced changes in the structural properties of TgFNR as studied by monitoring the changes in CD ellipticity at 222 nm, FAD fluorescence intensity and tryptophan fluorescence emission maxima with increasing urea concentrations. The curves obtained from these techniques showed a sigmoidal transition between 3.0 and 6.5 M urea suggesting that urea induces a two-state cooperative unfolding of TgFNR.

Figure 4B illustrates the effect of increasing concentrations of urea on the enzymatic activity of TgFNR. A sequential decrease in the enzymatic activity from 100% to 10% was observed between 0.0 and 3.0 M urea, however, at urea concentration above 4.0 M complete loss of enzymatic activity was observed. Consistent with the loss of enzymatic activity, a sequential decrease in FAD fluorescence polarization was also observed with increasing urea concentrations between 0.0 and 3.0 M (Fig. 4C). These observations collectively suggest that slight variations in the orientation of the protein bound FAD lead to loss of enzymatic activity (discussed in detail for GdnHCl).

The urea denaturation profiles as studied by monitoring the changes in enzymatic activity, FAD fluorescence polarization and intensity, tryptophan emission maxima and CD ellipticity at 222 nm with increasing urea concentrations were not superimposible. This suggests that urea-induced unfolding of TgFNR is also a multiphasic process. In the first phase, loss of enzymatic activity and decrease in FAD polarization was observed at low-urea concentrations (<3 M), which is followed by global unfolding of the protein at high-urea concentrations (between 3 and 6 M) as revealed by increase in FAD fluorescence, change in tryptophan emission maxima and decrease in CD ellipticity. However, during urea-induced denaturation of TgFNR no significant binding of the hydrophobic dye ANS was observed (Fig. 4D), which is in

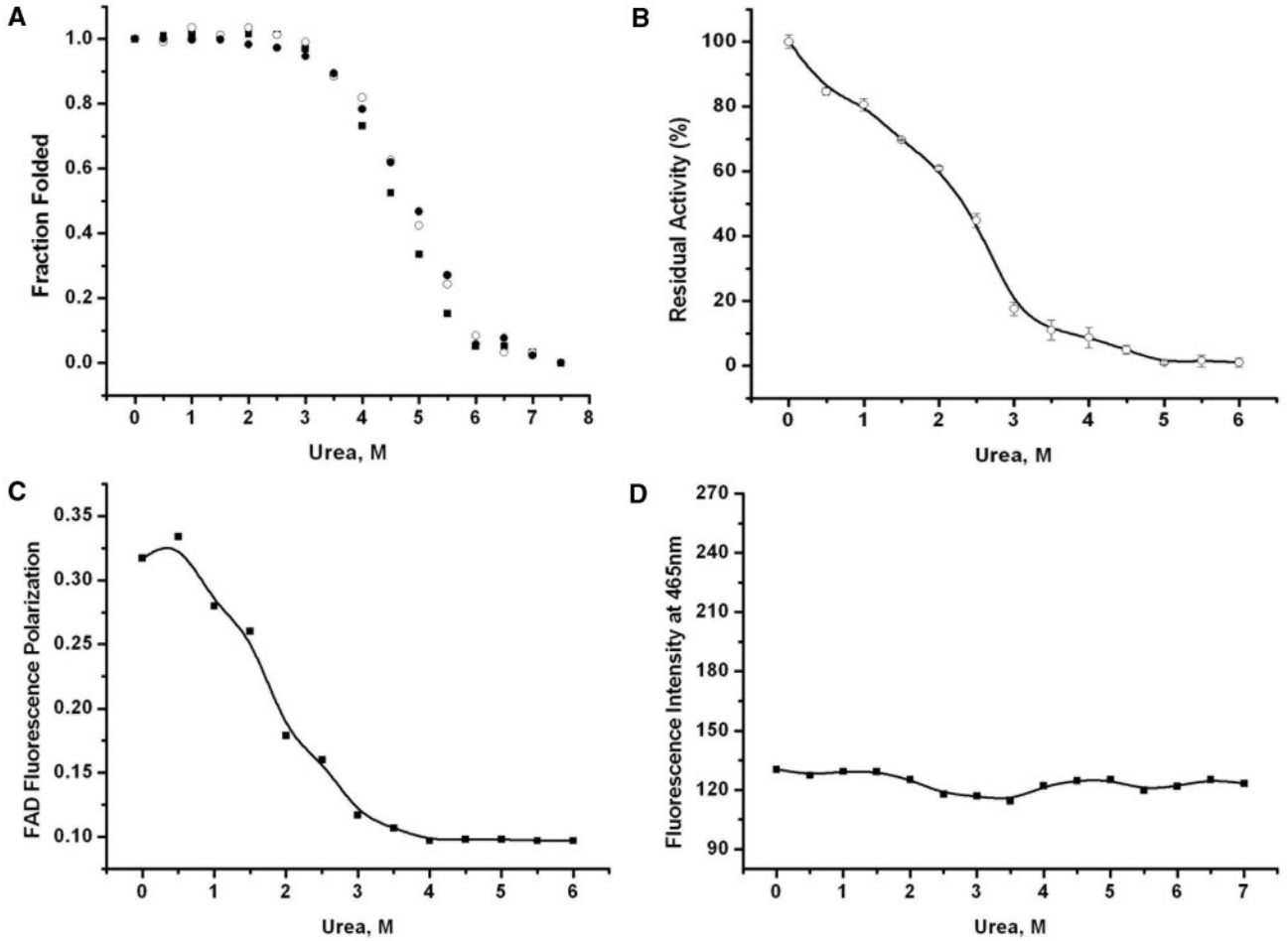

Fig. 4. **Changes in the molecular properties of TgFNR in the presence of increasing concentrations of urea at pH 7.5 and 25°C.** (A) Urea induced unfolding transitions of TgFNR as obtained from FAD fluorescence intensity (solid circle), tryptophan emission maxima (open circle) and CD ellipticity at 222 nm (solid square). The fraction of the native enzyme was calculated as described in Fig. 1A. (B) Changes in the enzymatic activity of the TgFNR on treatment with increasing concentrations of urea. The data are expressed as relative activity using the activity of native enzyme as 100%. The data represent as mean ± SD of three independent measurements. (C) Changes in FAD fluorescence polarization. (D) Changes in the fluorescence intensity of ANS on incubation with of TgFNR, treated with increasing concentrations of urea.

contrast to that observed for GdnHCl stabilized intermediate. The refolding efficiency of partially folded intermediate (obtained at 3 M urea) and unfolded of TgFNR (obtained at 8 M urea) was found to be 100% and about 70%, respectively (data not shown).

On SEC, the urea stabilized intermediate of TgFNR showed a retention volume of 10.03 ml, which is slightly higher than that observed for native enzyme indicating a slightly enhanced hydrodynamic radii for the urea stabilized intermediate of the enzyme (Fig. 5). However, the FAD cofactor was found to be associated with the enzyme under these conditions as the signals at 280 and 445 nm were found to be overlapping. For TgFNR treated with 7.0 M urea, a significant decrease in retention volume to 8.0 ml, indicating extensive unfolding of the enzyme along with dissociation of the FAD moiety was observed.

The structural cooperativity of TgFNR was also found to be affected by urea (Fig. 6A). Here, we mean that the structural cooperativity indicates the two-state thermal unfolding regardless of the slopes of the transition curves. Therefore, if it is the two-state, the molecule is cooperative as the unfolding takes place simultaneously in whole of the protein molecule (*19*). For TgFNR in the presence of 3 M urea, a single reversible transition corresponded to only about 38% loss of CD signals was observed. This demonstrates that at low concentration of urea, TgFNR undergoes only partial unfolding with major part of protein showing resistance to thermal denaturation. Hence, the structural cooperativity existing in native conformation of TgFNR is lost on treatment of protein with low concentration of urea. Furthermore, the Tm obtained in the presence of 3 M urea was lower (54°C) as compared to that for TgFNR at pH 7.5 (60°C) (Fig. 6A), supporting the fact that significant interactions existing between structural domains of the protein in native conformation were abolished in the presence of low concentration of urea.

Figure 6B shows loss of CD signals at 375 nm with increasing temperature. A sigmoidal loss of CD signals

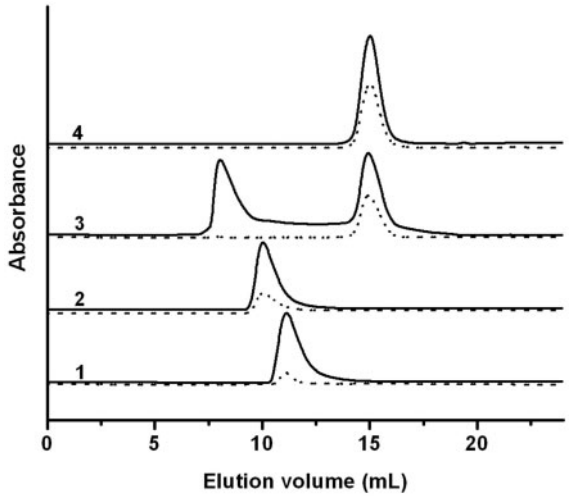

Fig. 5. **Urea induced changes in the molecular dimension of TgFNR during enzyme unfolding at pH 7.5 and 25°C.** SEC profiles on superdex 75HR column, profiles 1–4 represent sample of 0, 3 M, 7 M urea-treated TgFNR and FAD alone in 7 M urea, respectively. Solid and dotted lines represent absorbance at 280 and 445 nm, respectively.

under all experimental conditions was observed suggesting dissociation of FAD molecule from protein at higher temperature due to unfolding of FAD-binding domain. The T_m of about 60°C and 56°C was found to be associated with protein samples at 0.0 and 3.0 M urea concentrations, respectively. These Tms are close to those obtained for far-UV CD measurements for these samples under similar conditions. Hence, partial unfolding of TgFNR under these conditions also results in dissociation of FAD molecule from the protein, which can only happen when the FAD-binding domain undergoes unfolding under these conditions.

Proteolytic susceptibility of TgFNR to trypsin under native conformation as well as in the presence of 3 M urea were analysed (Fig. 6C). The pattern obtained for 3 M urea treated sample was very much similar to that obtained for GdnHCl stabilized intermediate mentioned earlier. Molecular mass and sequence analysis of proteolytic fragments suggest that $NADP^+$-binding domain undergoes modifications on interaction with low concentrations of urea and become resistant for proteolytic degradation (Table 1).

DISCUSSION

During folding/unfolding of most of the flavoproteins including FprA, apo intermediate of the enzymes are stabilized i.e. the FAD molecule is detached from the enzymes during the stabilization of intermediate (*19*, *35*, *36*). The equilibrium unfolding of TgFNR with GdnHCl and urea reported in this paper demonstrate that unfolding of the enzyme is a non-cooperative process with stabilization of a partially unfolded holo-intermediate (Fig. 7). Furthermore, although the FAD is bound to the enzyme in these intermediate conformations but no enzyme activity was observed.

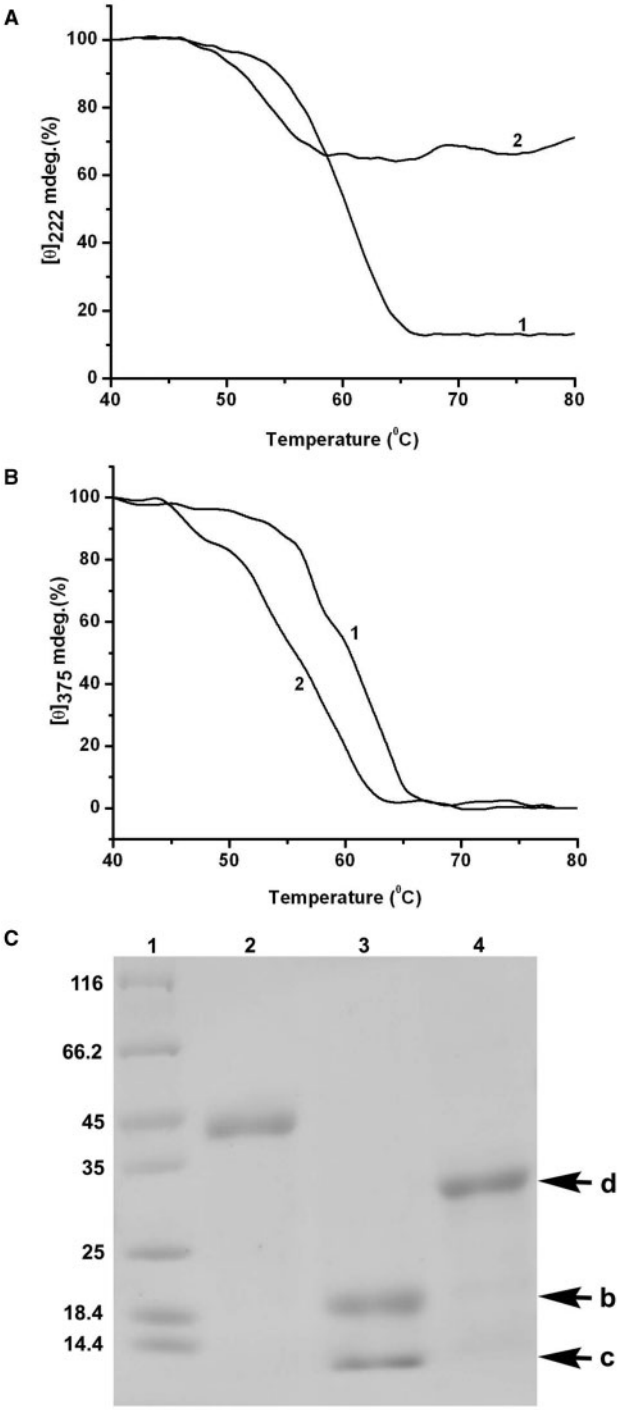

Fig. 6. **Thermal unfolding of TgFNR as studied by monitoring the loss of CD signals at 222 nm (A) and 375 nm (B) with increasing temperature.** The curves 1–2 represent samples of native and 3 M urea treated TgFNR, respectively. For both the panels A and B, data have been represented as percentage with the value observed for protein samples at 40°C taken as 100%. (C) Trypsin accessibility of urea stabilized intermediate, lanes 1–4 represent molecular weight marker, purified TgFNR, TgFNR in the absence and presence of 3 M urea after treatment with trypsin, respectively.

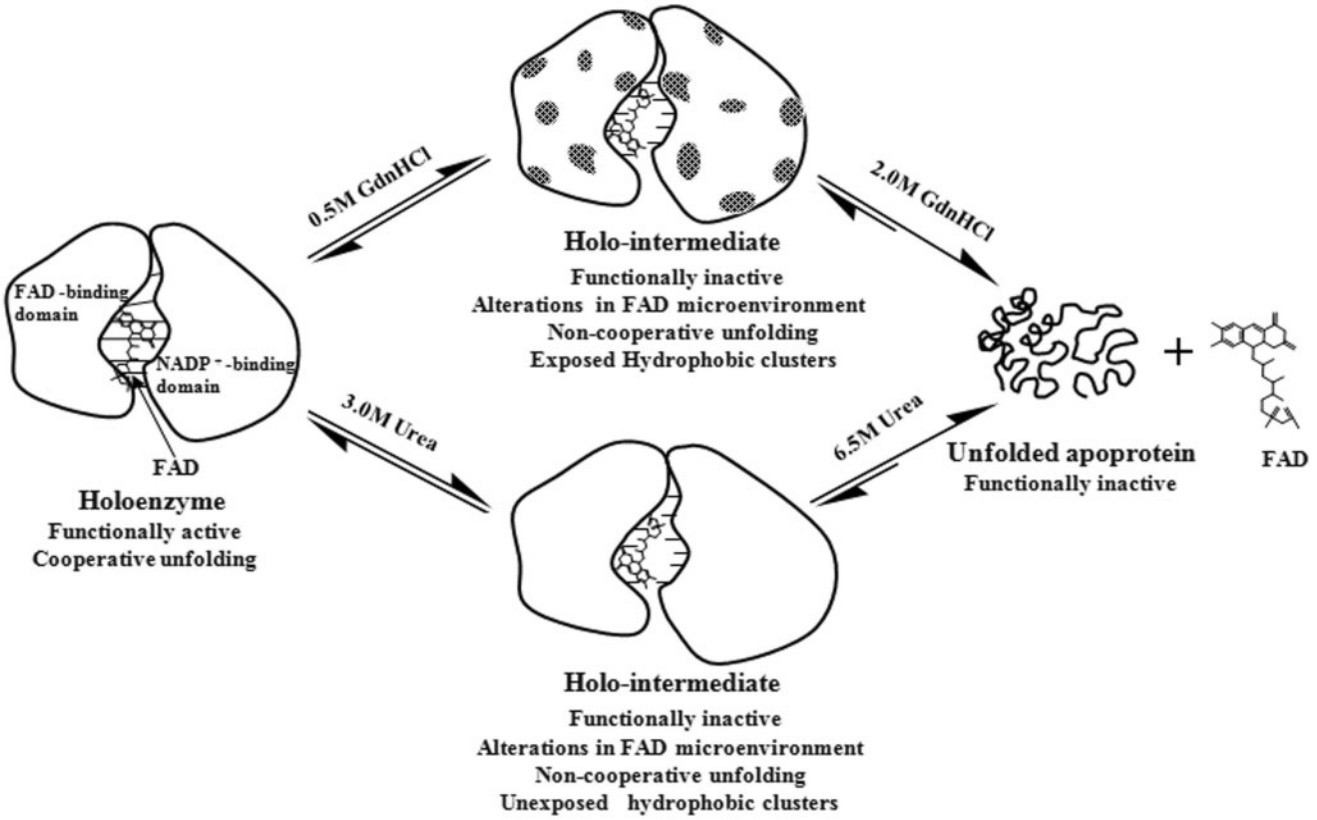

Fig. 7. **Schematic representation of the GdnHCl and urea induced unfolding of TgFNR.**

In FNRs, the FAD cofactor is tightly bound to FAD-binding domain with its isoalloxazine ring sandwiched between the FAD- and NADP⁺-binding domains (*10*, *17*). The overall binding mode of FAD is essentially conserved in almost all FNRs of known 3D structure and predominantly occurs through hydrogen bonding, van der Waal contacts and π–π interactions, which are essential for proper orientation of FAD cofactor and in turn necessary for optimum functioning of the enzymes (*10*). The loss of fluorescence polarization of FAD cofactor at low concentrations of GdnHCl and urea in TgFNR may be as a result of disruption of interactions of FAD with some amino acid side chains of the enzyme due to the denaturant-induced conformational changes under these conditions. This will affect the orientation of the FAD cofactor and subsequently the enzymatic activity is lost.

Recently, Lee *et al*. have suggested that NADP⁺-binding domain of maize FNR is divided into two sub-domains; NADP⁺-rigid and NADP⁺-flexible domains (*37*). The two sub-domains are connected by surface exposed flexible loop region that contains a protease active site. Due to this, the NADP⁺-binding domain is susceptible to proteolysis resulting in separation into two domains. On limited proteolysis of TgFNR with trypsin, two protein bands corresponding to Asn97-Lys279 and Asn280-Tyr355 were obtained, which demonstrated that NADP⁺-binding domain of TgFNR is also divided into two sub-domains similar to that of maize FNR. However, on treatment of enzyme with low concentrations of GdnHCl or urea, this cleavage site was found to be inaccessible to protease as no proteolysis of the enzyme was observed. This suggests that it is the NADP⁺-binding domain in the presence of low concentration of the denaturants that undergoes structural modifications such that the exposed proteolytic site present in native conformation of the enzyme gets buried and becomes inaccessible to protease. These observations clearly demonstrate that denaturants at low concentrations have preferential interactions with NADP⁺-binding domain of TgFNR, which lead to conformational changes of this domain for stabilization against thermal denaturation. At low-denaturant concentrations, GdnHCl and urea serve to crosslink different parts of the protein non-specifically at sub-global level by establishing multiple variable length hydrogen bonding and van der Waals' interaction with main-chain and side-chain groups of the protein (*38*, *39*). This reduces the motional freedom and induces stiffening in protein molecule/domain resulting in its enhanced stability against denaturation. It is possible that a similar phenomenon might be happening in NADP⁺-binding domain with denaturants at low concentrations.

Dynamic studies on maize FNR and thermal denaturation studies in FprA have shown that NADP⁺-binding domain is preferentially destabilized in low concentration of urea and GdnHCl, respectively (*19*, *37*). However, for TgFNR, stabilization of NADP⁺-binding domain in the presence of low concentrations of GdnHCl and urea were observed (Figs 3 and 6). This difference was found

to affect the structural cooperativity of FprA and TgFNR differently. The native FprA molecule shows a non-cooperative behaviour but in the presence of low-denaturant concentration it behaves cooperatively (*19*). However, for TgFNR at low concentrations of GdnHCl and urea, partial unfolding of the protein occurs, which result in induction of non cooperativity in otherwise structurally cooperative molecule (Figs 3 and 6). The loss of cooperative interactions between FAD- and $NADP^+$-binding domains may also result in stabilization of a slightly open conformation of the enzyme under these conditions, and this is probably the reason for enhanced hydrodynamic radii of the enzyme at 0.5 M GdnHCl and 3 M urea concentrations on SEC.

An interesting observation is that due to denaturant induced modifications in $NADP^+$-binding domain, interactions between $NADP^+$- and FAD-binding domains existing in the native TgFNR are lost, and this results in destabilization of FAD-binding domain (Figs 3A and 6A). This is evident from thermal denaturation studies (Figs 3A and 6A) and also supported by the fact that in the multi-domain proteins the inter-domain interactions stabilize the domain having lower Tm to a greater extent (*40*). Hence, in the native conformation of TgFNR, the enhanced stability of the FAD-binding domain seems primarily due to its strong interactions with $NADP^+$-binding domain. Interestingly, the destabilization of FAD-binding domain in the presence of GdnHCl was significantly greater as compared to that observed in the presence of urea. This difference is probably due to modulation of ionic interactions along with hydrophobic interactions by ionic denaturant GdnHCl as a similar induction of non-cooperativity and destabilization of FAD-binding domain in TgFNR was observed by us at pH 4.0 (*24*).

The studies presented in the paper demonstrate that a partially unfolded holo-intermediate of the enzyme is stabilized during denaturant induced unfolding of TgFNR. However, detailed structural analysis showed that the two intermediates were not structurally similar. The GdnHCl-stabilized intermediate had the presence of surface exposed hydrophobic patches as observed by ANS binding to the intermediate. In contrast, no exposure of hydrophobic clusters was observed for urea stabilized intermediate. Furthermore, even the hydrodynamic radius of the two intermediates was different. The GdnHCl-stabilized intermediate was more compact as compared to the urea-stabilized intermediate as observed by SEC (Figs 2 and 5).

Based on the studies presented in the article, it is clear that significant differences exist in the unfolding pathways of plant-type and GR-type FNRs.

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CONFLICT OF INTEREST

None declared.

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