

***Leishmania donovani* trypanothione reductase: Role of urea and guanidine hydrochloride in modulation of functional and structural properties[#]**

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Running head: Relation of function and structure in LdTR

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ABSTRACT

Trypanothione reductase [TR], an NADPH-dependent disulfide oxidoreductase, unique to kinetoplastid parasites including *Trypanosoma* and *Leishmania*, is a validated target for the design of improved drugs. TR is a stable homodimer with a FAD molecule tightly bound to each subunit. In this paper, structure, function, stability properties and cofactor protein interactions of recombinant TR from *Leishmania donovani* were investigated under equilibrium unfolding/denaturing conditions. Urea induced unfolding was non-reductive in nature and led to the formation of partially folded intermediate. This intermediate species lacks catalytic activity and characteristic conformation of native LdTR but has significant secondary structure and could be partially reactivated. Guanidine hydrochloride-induced irreversible denaturation was marked by the presence of molten globule intermediate. Reactivation and cross-linking experiments clearly demonstrated that the loss of activity at lower denaturant concentrations was not coincided by dimer dissociation or structural unfolding. The studies demonstrate that functional conformation and stability is largely governed by ionic interactions and active centre site disulphide plays a vital role in helping to maintaining functional conformation. The results obtained from this study provide intriguing insight into the possible mechanism/s of modulation of structure, function and stability of LdTR induced by the cationic, guanidine hydrochloride and the neutral denaturant, urea.

1. Introduction

Understanding the structure-function relationship of an enzyme under different conditions of denaturation is fundamentally important for theoretical as well as applicative aspects. Such studies provide insights into the molecular basis of stability of enzymes and also on the mechanism of modulation of enzyme function in response to changes in structure. Protein folding studies indicate a discrete pathway with the formation of intermediate states between native and denatured states [1, 2]. Analysis of the structural and thermodynamic properties of such intermediate states may provide an understanding of the factors involved in guiding the pathway of unfolding. One of these states, called the molten globule intermediate, is a compact globular molecule with substantial secondary structure and diminished tertiary structure [3] and is stable under mild denaturing conditions. The role of molten globule as a functional entity in protein folding is hypothesized, and further evidence has also shown that such a state is involved in several biological processes such as membrane insertion, trans-membrane trafficking, and chaperone-assisted refolding that require the protein to be partially unfolded [4].

Leishmania parasites cause a wide spectrum of human and animal infections ranging from the life threatening visceral disease to the disfiguring mucosal and cutaneous forms of the disease. These neglected diseases continue to pose a major threat to human health and economic development worldwide [5]. Treatment of leishmaniasis relies mainly on chemotherapy and is far from ideal because of high costs, high toxicity and long term treatment requirements [6,7]. Increasing incidences of therapeutic failures [8,9] and emergence of drug resistant parasites [10,11], now warrants an urgent need for new improved drugs. Characterization of kinetoplastid parasites has suggested biochemical pathways sufficiently different from human metabolic pathways where chemical intervention may prove a possible route to control the infection. One such pathway is the maintenance of thiol redox balance [12]. Unlike their insect and mammalian hosts, the kinetoplastid parasites including Leishmania lack glutathione reductase (GR) and use trypanothione (N1, N8-bis (glutathionyl) spermidine) and trypanothione reductase to regulate an intracellular reducing environment. TR is a member of flavoprotein oxidoreductase family, homodimer with a subunit molecular weight of approximately 52 kDa. It catalyses transfer of electrons from NADPH to oxidized trypanothione via FAD prosthetic group and a redox active cysteine disulfide [13,14]. TR is vital for intracellular parasite survival [15,16] and infectivity [17], which makes this pathway an excellent target against Leishmania.

The crystal structure of TR [18,19] as well as its complex with substrate [20,21] and inhibitor [22,23] are known. The protein comprises three domains namely the FAD-binding domain, the NADPH-binding domain and the C-terminal interface domain [19]. The N-terminal region adopts the Rossmann fold which represents the dinucleotide-binding motif [24]. The substrate binding cleft is formed at the dimer interface and the active site residues are contributed by both the identical subunits.

TR of *Leishmania donovani* (LdTR) has already been cloned and expressed in our lab [25]. To understand the mechanism of modulation of enzyme function in relation to the changes in structure, we carried out detailed structural, functional and stability studies on recombinant LdTR in the presence of neutral and cationic denaturants.

2. Materials and methods

2.1. Materials

Trypanothione (TS₂) was purchased from Bachem, Switzerland. Glutathione sepharose-4B resin and thrombin were purchased from GE Healthcare Bio-Sciences Ltd. Centricons were purchased from Ambion. All other chemicals of the highest purity were purchased from Sigma Chemical Co.

2.2. Protein expression and purification

LdTR was expressed in *E. coli* BL21 (DE3) and purified to homogeneity as described earlier [25]. Purity of the recombinant enzyme was checked on a Superdex 200HR 10/30 column (manufacturer's exclusion limit of 600 kDa for proteins) on AKTA-FPLC (Amersham Pharmacia Biotech.) and by SDS-PAGE [26].

2.3. Assay of enzyme activity

Enzyme activity was measured according to the method of Hamilton *et al.* [27]. Briefly, the reaction mixture contained 40 mM HEPES pH 7.5, 1 mM EDTA, 0.15 mM NADPH, 25 μ M DTNB and 1 μ M TS₂. The reaction was initiated by the addition of oxidized trypanothione and the change in O.D. was monitored at 412 nm.

2.4. Circular dichroism measurements

CD measurements were made with a Jasco J800 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results are expressed as mean residual ellipticity $[\theta]$, which is defined as $[\theta] = 100 \theta_{\text{obs}}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, c is concentration in moles of residue per litre, and l is the length of the light path in centimeters. The CD spectra were recorded at an enzyme concentration of 0.7 μ M in 10 mM sodium phosphate buffer, pH 7.5 with a 2 mm path length cell at 25°C. The spectra obtained were corrected for background contribution by subtracting the baseline recorded for the buffer having the same concentration of salts (urea and GdmCl) under similar conditions. Each spectrum represents the mean of two scans. Far-UV CD spectra were run in the range of 200-250 nm at a scan speed of 50 nm min⁻¹. Protein denaturation was monitored by recording the changes in $[\theta]$ at 222 nm. Secondary structure content of native LdTR was calculated using the DICHROWEB web site at Birbeck College [28,29] using the CDSSTR, CONTIN and SELCON3 methods.

2.5. Fluorescence Spectroscopy

Fluorescence spectra were recorded with a Perkin Elmer LS 50B fluorescence spectrometer in a 5 mm path length quartz cell at 25°C. Experiments were carried out at room temperature in 10 mM sodium phosphate buffer pH 7.5 using 0.6 μ M protein. Tryptophan fluorescence was monitored by excitation at 290 nm and emission recorded between 300 and 400 nm whereas FAD fluorescence was studied by exciting LdTR at 460 nm and recording emission spectra between 480 and 600 nm. FAD dissociation studies were carried out by concentrating the treated samples on a centricon of 10 kDa cutoff and estimating the fluorescence in the free form (filtrate) as well in the enzyme bound form (protein fraction). The spectra obtained were corrected for background contribution by subtracting appropriate blank containing buffer having the same concentration of salts (urea and GdmCl) under similar conditions.

2.6. Absorbance spectra

Absorbance spectra of the oxidized and NADPH-reduced LdTR were measured with Ultrospec 3100 UV-Vis spectrophotometer from GE Healthcare Bio-Sciences Ltd in the wavelength range of 220 nm to 600 nm in a 10 mm path length cell.

2.7. 8-Anilino-naphthalene 1-sulfonic acid binding experiments

ANS binding to LdTR was estimated at 50 μM final concentration of ANS and 3 h of incubation at 25°C. The protein concentration and buffer conditions were same as described for intrinsic fluorescence. The excitation wavelength was 380 nm and emission was recorded between 400 and 600 nm.

2.8. Fluorescence quenching experiments

Acrylamide quenching experiments were performed by titrating an 8 M acrylamide stock solution into samples of LdTR (native condition and after 1 h treatment with GdmCl and urea). Fluorescence quenching data were analyzed according to the Stern Volmer equation:

$$F_0/F = 1 + K_{sv}[Q]$$

Where, F_0 and F are the fluorescence intensities in the absence and presence of acrylamide. K_{sv} is the quenching constant and $[Q]$ is the molar concentration of acrylamide.

2.9. Chemical denaturation studies

Chemical denaturation studies were performed by incubating the enzyme in 10 mM sodium phosphate buffer containing increasing concentrations of denaturants (inactivation mixture) for 1 h at 25°C followed by enzyme assay, CD and fluorescence spectra in the presence of denaturant at respective concentrations. The kinetics of inactivation was studied by taking aliquots from the inactivation mixture at defined time intervals (0, 15, 30, 60, 120, 240, 300 and 360 min) and assayed the enzyme activity. The enzyme was found to attain complete inactivation in 1 h and further increase in incubation period had no significant effect on residual activity. Hence the incubation time for chemical denaturation was kept 1 h.

2.10. Dimethyl suberimidate crosslinking of LdTR

Crosslinking of native and denatured LdTR was performed at a protein concentration of 0.2 mg/ml in the presence of 20 μM dimethyl suberimidate dihydrochloride [30]. The reaction mixture contained protein in 200 mM triethanolamine hydrochloride buffer pH 8.5 and incubated for 4 h at 25°C. LdTR was denatured in GdmCl and urea for 1 h at room temperature before cross-linking. The cross-linked protein was then precipitated by adding TCA and resolved on 10 % SDS-PAGE.

2.11. Renaturation/Reactivation experiments

To check the reversibility of the transitions, LdTR in 20 μl aliquot was first incubated for 1 h in presence of the different concentrations of denaturants. The samples were then diluted with 10 mM sodium phosphate buffer pH 7.5 to attain the final concentration of urea to 10 mM and GdmCl to 2.5 mM respectively. The diluted enzyme mixture was then immediately used for trypanothione reductase activity. These residual concentrations of urea and GdmCl in the reactivation mixture had negligible effect on the activity of LdTR. The reactivation of LdTR after 24 h of denaturant treatment also resulted in the similar levels of regained activity.

2.12. Sulfhydryl group estimation

The numbers of DTNB-titratable thiols in the enzyme were determined under conditions of reduction and denaturation. The reaction mixture contained 3.75 μM LdTR, 0.1 M Tris pH 8.2 and 1 mM EDTA. DTNB was added to a final concentration of 0.1 mM and increase in absorbance at 412 nm was monitored. An extinction coefficient of 13.6 $\text{mM}^{-1}\text{cm}^{-1}$ [31] was used for stoichiometric determinations. The titration of thiols in reduced enzyme

was monitored by treating LdTR with 0.3 mM NADPH for 10 min. Thiol titration of oxidized and reduced enzyme was performed after 1 h treatment with urea and GdmCl.

2.13. Data analysis

The thermodynamic properties of LdTR were calculated assuming a two state denaturation process. Unfolding curves for the N \leftrightarrow D transition were normalized to the apparent fraction of the unfolded form, F_D , using the following equation [32],

$$F_D = (Y - Y_N) / (Y_U - Y_N) \quad (\text{Eq. 1})$$

where Y is the observed variable parameter, and Y_N and Y_U are the values characteristic of the native and fully unfolded conformations (at highest concentration of denaturant used) respectively. The difference in free energy between the folded and the unfolded state, ΔG , was calculated by the following equation,

$$\Delta G = -RT \ln K = -RT \ln [F_U / (1 - F_U)] \quad (\text{Eq. 2})$$

where K is the equilibrium constant, R is the gas constant, and T is the absolute temperature. The data were analyzed assuming the free energy of unfolding, ΔG , to be linearly dependent on the denaturant concentration (denoted here by C), as described in detail previously [33],

$$\Delta G = \Delta G_w - mC = m(C_m - C) \quad (\text{Eq. 3})$$

where ΔG_w and ΔG represent the free energy of unfolding in the absence and presence of denaturant, respectively; C_m is the midpoint concentration of denaturant required for unfolding; and m stands for the slope of the unfolding curve at C_m .

3. Results

3.1. Structural features of native enzyme

Fig. 1A depicts the Far UV CD spectrum of LdTR under native conditions, which is characteristic of an α/β protein. Table 1 enlists the secondary structure content calculated by CDSSTR, CONTIN and SELCON3 methods. The helix content was calculated as 26, 22.7 and 25.5 % whereas percent sheets were 26, 20.4 and 19.5 % respectively. Tryptophan, the dominant intrinsic fluorophore, is very sensitive to its local environment. Exposure of the tryptophan residues to the polar environment results in a characteristic red shift [34]. Hence steady state tryptophan fluorescence has been extensively studied to explore the structural and dynamic properties of proteins. LdTR possesses six tryptophan residues per monomer at positions 21, 80, 91, 134, 135 and 147. LdTR suspended in 10 mM sodium phosphate buffer pH 7.5 when excited at 290 nm, exhibited emission spectrum with λ_{max} at 334 nm (Fig. 1B) corresponding to the average fluorescence of the six tryptophan residues of the protein monomer. LdTR intrinsic fluorescence suggests that all the six tryptophan residues in the monomer are located in non polar region of the protein. Recombinant LdTR when excited at 460 nm, exhibited emission spectra with peak at 525 nm showing the presence of FAD group (Fig. 1C). According to the *T. cruzi* TR structure, FAD molecule is tightly bound to the N-terminal domain through hydrogen bonds and hydrophobic interactions. Residue Phe 199 in the absence of NADPH, covers the isoalloxazine ring system and protects the molecule from aqueous environment [19]. Hence in the native state of LdTR, weak fluorescence intensity of FAD was observed for emission at 525 nm. Further absorption spectrum of LdTR, in the wavelength range 220 to 600 nm, showed peaks at 281, 386 and 466 nm which is similar to that observed for TR from *L. infantum* and *C. fasciulate* [35,36]. Fig 1 D shows the spectrum of native enzyme (curve a) and curve b represents the spectrum after reduction with 2 molar excess of NADPH. It is evident from the observation that the peaks at 386 and 466 nm significantly decreased after reduction with NADPH.

3.2. Effect of urea on the structural and functional properties of LdTR

3.2.1. Activity is lost prior to any detectable change in secondary structure

Fig. 2A exhibits the effect of increasing concentrations of urea on enzyme activity of LdTR. 1 mM urea had no significant effect on activity whereas 50 % enzyme inhibition was observed at 750 mM concentration. Further inactivation by urea is independent of LdTR concentration (0.025, 0.05 and 0.1 $\mu\text{g/ml}$), suggesting that LdTR did not dissociate during the process. Though the enzyme activity was completely lost at 3 M urea, but at this concentration no significant change was observed in ellipticity at 222 nm (Fig. 2B). 4 M urea induced structural unfolding as CD ellipticity decreased at 222 nm. Interestingly, treatment with 8M urea resulted in only ~50 % loss of relative helical content as compared to the native state (Fig. 2B). The transition midpoint was calculated as 5.5 M taking 8M transition as end point. Thus, the plot of changes in CD spectra as a function of urea concentration apparently suggests a simple two state transition. The conformational stability of the protein as calculated by enzyme activity and secondary structure measurements was 0.61 ± 0.05 kcal/mol and 1.95 kcal/mol respectively. The respective *m* values were 0.926 and 0.35.

3.2.2. Changes in protein fluorescence indicate the presence of intermediate species at lower concentrations

The tryptophan fluorescence emission maxima showed a gradual shift in the wavelength with respect to increasing concentrations of urea (Fig. 2C) whereas fluorescence intensity showed an initial increase till 3 M urea and registering a decrease at 4 M and above. Urea below 3 M caused a minor red shift (~1 nm) whereas major structural changes were induced only at 4 M and reached a plateau at 7 M. Complete denaturation in 8 M urea was marked by a red shift of 14 nm suggesting that the tryptophan residues experienced significant solvent exposure as the polypeptide was unfolded considerably. ΔG_{U-N} was 1.85 kcal/mol and *m* value was 0.369.

3.2.3. Urea induced increase in flavin fluorescence is due to FAD dissociation

In accordance to earlier observations, urea below 3 M did not cause any significant alteration in flavin fluorescence curve (Fig. 2D). Major change in the protein conformation was induced at 4 M as the FAD fluorescence was marked by an increase in intensity. The fluorescence intensity reached its maximum value at 8 M urea indicating extensive exposure of FAD to the solvent. FAD dissociation studies revealed that 4 M urea-denatured enzyme retained ~ 59 % relative fluorescence and the remaining 41 % relative fluorescence was observed in the free form indicating that the observed increase in FAD fluorescence at 4 M was due to dissociation of the FAD group from the protein. At 6 M urea, the fluorescence in the enzyme bound form was completely lost which implies that the FAD group was completely dissociated due to major changes in the enzyme conformation. The value of ΔG_w calculated from the curve was 1.8 kcal/mol and *m* value was 0.301.

3.2.4. Presence of intermediate species was confirmed by ANS binding

The urea denaturation profile of LdTR as studied by monitoring the changes in enzymatic activity, ellipticity at 222 nm, tryptophan and FAD fluorescence depicts a non-superimposable profile (Fig 2E), suggesting the existence of folding intermediates. To confirm the presence of folding intermediates, binding characteristics of urea treated LdTR towards the hydrophobic probe 1, 8 anilino –naphthalene sulfonate (Fig. 3A) were examined. The fluorescent probe ANS, binds to the solvent accessible clusters of non-polar groups in proteins; the fluorescence emission of the probe is known to increase on binding to the hydrophobic clusters of proteins [37]. Urea at ≤ 3 M concentration led to a decrease in ANS

fluorescence as compared to the native protein. It is clearly evident from the above data that urea concentration up to ≤ 3 M induced some changes in the hydrophobic patches of the protein while, 4 M urea led to a significant increase in emission intensity. At higher concentration (6 – 8 M), LdTR bound ANS fluorescence was marked by a decrease in emission intensity with a simultaneous blue shift in emission maxima.

3.2.5. Unfolding Intermediate did not show quenching of tryptophan fluorescence

In order to probe the water accessibility of protein core, quenching of fluorescence intensity of tryptophan residues under conditions of urea denaturation was studied. Urea at ≤ 4 M did not cause any significant change in the level of fluorescence quenching while the tryptophan fluorescence was quenched to a significant extent in 8 M urea- denatured LdTR (Fig. 3B). The values of the Stern-Volmer quenching constants for the urea denatured states are listed in Table 2

3.2.6. Active site disulfide bond remains intact under urea denaturation

To investigate the parameters responsible for the high stability and reversion of partial enzymatic activity in 8 M urea, the protein thiol groups were titrated. Native LdTR showed 1.22 titratable –SH groups whereas NADPH reduction raised the titer to 3.26 (Fig. 3C). The increase of two –SH groups was presumably from the active centre disulfide bond. 8 M urea treated protein possessed 3.94 –SH groups, however reduced enzyme denatured with 8 M urea displayed a titer of 7.9. This clearly suggests that in presence of 8 M urea, the active site disulfide remained intact which on NADPH reduction, caused an increment of two in the titer. The other two groups are probably detected due to further unfolding of the reduced enzyme.

3.2.7. Reactivation experiments establish that urea-induced unfolding is reversible

Renaturation or reactivation of LdTR after treatment with urea is summarized in Fig. 3D. LdTR after treatment with urea below 500 mM regained > 95 % activity. This reactivation of the enzyme suggests that lower concentrations of urea resulted in minor, completely reversible structural changes, probably in and around the active site which could be easily reverted upon dilution of the denaturant. Even at 2 M concentration of urea, 90% activity was regained. At 5.5 M urea concentration (at which half of the secondary structure was lost; Fig 2B), 70 % activity was resumed. Interestingly, 8 M urea treated LdTR could also be reactivated up to 50 % of native activity.

3.2.8. Coupled to unfolding are changes in associative behavior

Fig. 4 shows the dimer/monomer conversion of LdTR upon urea denaturation as revealed by cross linking experiments. Under the present set of experimental conditions native LdTR did not show 100 % cross linking and $\sim \leq 25$ % of un-crosslinked monomers were observed on SDS-PAGE. The protein however, exists entirely as dimer as confirmed by the size exclusion chromatographic profile (data not shown). Hence the cross-linking error was neutralized from the data during analysis. Urea up to 4 M concentration did not significantly disturb the association state of the protein, whereas 8 M urea shifted the equilibrium towards dimer dissociation. Spot densitometric analysis revealed that 6 M urea treatment led to ~ 50 % dissociation whereas 8 M urea induced $\sim 60\%$ dissociation of the dimer as compared to the native state where dimerization was taken as 100 %.

3.3. Effect of the ionic modulator guanidinium hydrochloride on structure and function

3.3.1. Modulation of enzyme activity in relation to secondary structure

A sharp decrease in residual enzyme activity was observed with increasing concentrations of GdmCl (Fig. 5A). Inactivation of LdTR by GdmCl was found to be independent of LdTR concentrations (0.025, 0.05 and 0.1 $\mu\text{g/ml}$), suggesting that LdTR did not dissociate during the process. Though, the enzyme activity was completely lost at 200 mM GdmCl but no change in CD ellipticity was observed up to 1 M GdmCl (Fig. 5B). After that, an exponential decrease in CD ellipticity at 222 nm was observed along with increasing concentrations of GdmCl. At 4.5 M concentration, the signal at 222 nm was completely lost indicating extensive or complete unfolding of LdTR. Thus GdmCl induced structural transition is highly cooperative as is evident from the sigmoidal single step denaturation profile with a transition midpoint at 2.4 M.

3.3.2. Changes in tertiary structure as measured by protein and flavin fluorescence

Tryptophan fluorescence emission maxima exhibited a gradual red shift with increasing concentrations of GdmCl (Fig. 5C). Below 1 M, GdmCl caused a minor shift of 1 nm, however significant denaturation was observed at 1.5 M concentration which reached a plateau at 3 M. GdmCl denaturation below 2 M led to a gradual decrease in emission intensity (Fig 5C), whereas an increase in intensity was registered at 2.5 M and above. A pronounced red shift of 20 nm was observed on complete denaturation at 6 M GdmCl concentration. These observations suggest that treatment of LdTR with increasing concentrations of GdmCl lead to the exposure of buried tryptophan residues towards the solvent due to unfolding of enzyme molecule. The biphasic curve of fluorescence intensity indicated the presence of intermediate species.

Accordingly, no change in FAD fluorescence emission intensity at 525 nm occurred up to 1 M GdmCl (Fig. 5D). Gradual sigmoid increase was induced at 1.5 M GdmCl. Pattern of FAD fluorescence emission suggests that the protein conformation upto 1 M GdmCl is very similar to that of native protein and hence binds the FAD group tightly. Further increase in GdmCl concentration results in protein unfolding and exposure of FAD molecule to solvent. FAD dissociation studies revealed that 2 M GdmCl caused significant dissociation of FAD, as 55% relative fluorescence was observed in the filtrate and the rest (45 %) in the enzyme bound form. At 3 M concentration, 87 % relative fluorescence was observed in the filtrate and enzyme bound fraction retained 13 % only. 6 M GdmCl treatment resulted in complete dissociation of FAD as the fluorescence signal in the enzyme bound fraction was completely abolished. These observations indicate that GdmCl-induced structural transition is accompanied by loss of bound cofactor resulting in increase in FAD fluorescence intensity at 525 nm.

3.3.3. Exposure of hydrophobic patches indicate molten globule intermediate

Though, changes in the molecular properties of LdTR such as CD ellipticity at 222 nm, tryptophan and FAD fluorescence at increasing GdmCl concentrations showed concentration dependence but the profiles were not super-imposable (Fig. 5E) which suggests that the GdmCl-induced unfolding of LdTR is a multiphasic process with stabilization of intermediates. To experimentally evaluate the above suggestion, exposure of the hydrophobic patches was studied by measuring ANS binding to LdTR in presence of GdmCl (Fig. 6A). The fluorescent compound ANS binds specifically to clusters of hydrophobic groups, which are highly indicative of molten globule state [38,39]. Binding of ANS to hydrophobic clusters results in an increase in fluorescence. For most of the proteins

investigated in this respect, conversion to the molten globule state but not to the fully unfolded state, results in an increase in ANS fluorescence [38,40]. Expectedly, it was observed that GdmCl concentrations upto 2 M led to a gradual increase in intensity of emission of ANS bound to LdTR, with the emission maxima at ~455 nm as observed for the native state. The increment in emission intensity of ANS reached maximum level at 2.5 M GdmCl accompanied by a shift of emission maxima to ~442nm. Further increase in GdmCl concentration was marked by a decrease in emission intensity of protein bound ANS. The most significant observation was the differential ANS binding capacity of LdTR denatured with 1M GdmCl as compared to the native state. Thus it is clear that GdmCl treated LdTR possesses different measures of exposed hydrophobic areas in presence of different concentrations of GdmCl. Below 1 M GdmCl, though the spectroscopic probes of CD and FAD fluorescence did not highlight any difference in the properties of native and denatured LdTR, but tryptophan fluorescence and ANS binding clearly indicate the existence of at least one denatured intermediate between 0.5 and 1 M GdmCl concentration with properties similar to the molten globule (MG) state. Further, ANS binding experiments clearly indicate the presence of a second intermediate species at 2.5 M GdmCl possessing maximum fluorescence emission intensity and a sharp decrease in wavelength of emission (Fig 6A).

3.3.4. MG intermediate exhibits increased fluorescence quenching

Another characteristic of molten globule states is a significant increase in the hydrodynamic radius as a result of penetration of water in the folded core of the protein. Differential ANS binding characteristics of LdTR treated with lower concentrations of GdmCl (< 1 M) prompted us to investigate such solvent penetration by measuring the quenching of intrinsic fluorescence from aromatic side chains under conditions of GdmCl denaturation by acrylamide. LdTR treated with 0.5 M and 1 M GdmCl exhibited a marked increase in fluorescence quenching (Fig. 6B) as compared to quenching in native state demonstrating increased interaction of the buried aromatic side chains with the solvent. Expectedly, the fluorescence was quenched to a greater extent in 2.5 M GdmCl denatured LdTR. The Stern-Volmer quenching constants for the GdmCl denatured states are mentioned in Table 2.

3.3.5. Unfolding is complete at 4 M GdmCl

Sulfhydryl titrations were carried out for GdmCl denatured protein (Fig. 6C). Interestingly, in 2.5 M GdmCl treated enzyme, 4.02 –SH groups were detected whereas after 4 M GdmCl treatment, titratable –SH groups were 6.47. The denaturation of reduced LdTR with either 4 M or 6 M GdmCl did not alter the –SH titre any further. Thus it is evident from the above data that on increasing the concentration of GdmCl, there was a gradual exposure of protein thiol groups and denaturation reached a plateau at 4 M GdmCl which was marked by the exposure of all protein thiol groups. Surprisingly, we could not experimentally demonstrate the GdmCl concentration which leads to the disruption of the active site disulfide bond as our attempts to reduce the oxidized denatured enzyme failed even at lowest concentration i.e. 100 mM. No explanation could be offered for this observation at this moment.

3.3.6. GdmCl induced unfolding is an irreversible process

To check the reversibility of the GdmCl- induced transitions, reactivation of LdTR enzyme activity was studied. As shown in Fig. 6D, LdTR treated with GdmCl below or at 200 mM concentration regained > 90 % of enzyme activity. An increase in concentration up to 1 M resulted in 80 % gain of activity while only 40 % activity could be recovered after 2.5

M GdmCl treatment. 4 M GdmCl-treated protein could not be reactivated. These observations suggest that GdmCl below 200 mM induces minor reversible changes, probably in and around the active site, without any gross changes in enzyme structure due to which reactivation is possible upon dilution of the denaturant. The inability of LdTR to reactivate after denaturation with higher concentrations of GdmCl has resulted from the inability of the unfolded intermediate to refold and recover the dissociated prosthetic group due to irreversible conformational changes.

3.3.7. Unfolding is coupled to dimer dissociation

To investigate the association behavior of LdTR after treated with different GdmCl concentrations, SDS-PAGE analysis of the untreated and GdmCl-treated crosslinked protein samples was carried out (Fig. 7). Spot densitometric analysis, taking native untreated TR as 100 % dimeric form, revealed a shift of the equilibrium towards the dissociation of dimer with increasing concentrations of GdmCl. 1.5 M GdmCl led to ~ 25 % dissociation whereas at 2.5 M GdmCl dimer dissociation was ~ 56 %. 4 M GdmCl treatment led to > 93 % dissociation.

3.3.8. Quantitative estimation of GdmCl induced transitions

The above results suggest that GdmCl induced denaturation is multiphasic and involves at least two dimeric intermediates (I_{MG} and I_G) and two reversible steps $N \leftrightarrow I_{MG}$ and $I_{MG} \leftrightarrow I_G$. Each of the two steps was analyzed according to a two step equation (details in materials and methods). The free energy change and the denaturant m value associated with the first reversible step i.e., inactivation were 0.44 ± 0.015 kcal/mol and 16.66 ± 0.37 . The free energy change of the second reversible step i.e., $I_{MG} \leftrightarrow I_G$ estimated from CD, protein and flavin fluorescence were 1.35, 0.7 and 1.2 kcal/mol respectively. The corresponding m values were 0.594, 0.322 and 0.41.

3.3.9. Effect of guanidine hydrochloride on urea denaturation

To establish that urea induced LdTR denaturation is incomplete, we titrated 8 M urea denatured LdTR with GdmCl. The samples were then assayed for reactivation and CD analysis. Fig 8 A shows the reactivation profile of 8 M urea denatured LdTR titrated with increasing concentrations of GdmCl. It is clearly evident that addition of 100 mM GdmCl has no effect on reactivation, however 250 mM GdmCl inhibits reactivation by 50 %. Interestingly, reactivation is completely inhibited by 750 mM GdmCl. CD spectral analysis also exhibited the same profile (Fig 8B), where addition of 500 mM GdmCl further unfolded the urea denatured intermediate. Treatment with 1 M GdmCl resulted in complete loss of secondary structure of 8 M urea denatured intermediate. Though 750 mM GdmCl could inhibit the reactivation of 8 M urea intermediate almost completely, but complete structural unfolding was obtained only at 1 M concentration of GdmCl.

4. Discussion

Differential effects of guanidine hydrochloride and urea on the structural, functional and stability properties of LdTR provide a comparative analysis of the mechanism of modulation of enzymatic activity by the interplay of ionic and hydrophobic interactions. Though the exact molecular mechanism/s of the denaturing action of urea and GdmCl has not yet been defined [41, 42] but it has been presumed that both urea and GdmCl unfold proteins by solubilizing the non-polar parts of the protein molecule along with the peptide backbone CONH groups and the polar groups in the side chain of the proteins [43,44].

The equilibrium denaturation of dimeric LdTR in urea and GdmCl suggests different mechanisms of unfolding as summarized in Fig. 9. The results obtained by the urea –induced

denaturation of LdTR clearly indicate that the native-denatured transition is significantly reversible and is multiphasic. Urea at ≤ 3 M concentration resulted in complete loss of enzyme activity and induced formation of a native-like intermediate (I_{U1}) with disturbed hydrophobic interactions, as suggested from a red shift of 1 nm in tryptophan fluorescence, a decrease in fluorescence intensity at 455 nm in ANS binding, no change in acrylamide quenching and 90% recoverable enzyme activity. At higher concentrations of urea i.e., > 4 M, inactive LdTR can be partially reactivated and this transition is overlapped with both changes in the secondary structure and gross changes in conformation. The unfolding transitions do not reveal any accumulation of intermediate states at increasing urea concentrations but culminates into the formation of a denatured state that is a partially folded intermediate (I_{U2}) which possesses 50 % secondary structure and 50 % dimer population with intact active site disulfide bond (C52-C57) that can refold back to a partial functional conformation (Fig. 3 C,D). These observations suggest that the disulfide bridge is a dominant contributor to the overall functional stability of LdTR. Further, non-denaturing concentration of GdmCl (1 M) was sufficient to unfold the urea intermediate (I_{U2}).

In presence of GdmCl, the denaturation process is irreversible in which the unfolding and dimer dissociation are coupled. GdmCl-induced unfolding apparently occurs in at least two stages, and the first stage is associated with loss of activity (≤ 0.2 M GdmCl) and sufficient opening of LdTR structure that leads to the formation of a molten globule (MG)-like intermediate state (at 0.5 and 1 M GdmCl). GdmCl-induced MG (I_{MG}) state is characterized by the presence of native-like secondary structure, flavin fluorescence emission and dimeric association. However, the tryptophan fluorescence is slightly red shifted (1 nm) which is accompanied by enhanced ANS fluorescence intensity and tryptophan fluorescence quenching (Fig. 6A,B). Thus it is confirmed that I_{MG} state has well ordered secondary structures but the tertiary structure is perturbed due to an increase in hydrodynamic radius and exposure of hydrophobic patches. It was reported for yeast GR that low concentrations of GdmCl (≤ 1 M) induces subtle conformational changes as judged by the increased reactivity of thiol group to DTNB without any gross change in spectroscopic properties of CD and fluorescence [45].

Further increase in the concentrations of GdmCl led to a progressive unfolding of structure and conformation and a second intermediate state (I_G) is populated around 2.5 M GdmCl. Our studies confirm that the unfolding intermediate observed at 2.5 M GdmCl has exposed hydrophobic patches on structural sites that are accessible to ANS, as the ANS fluorescence intensity recorded a maximum at this concentration (Fig. 6A). Fluorescence quenching by acrylamide also registered a 2- fold increase as measured by quenching constant, K_{sv} . Interestingly, the structural characteristics of I_G closely resemble those of the urea denatured intermediate I_{U2} .

Taken together, the overall results of the structural characterization of LdTR are noteworthy. We for the first time demonstrated that inactivation of LdTR at lower concentrations of denaturants are not accompanied by unfolding rather by minor structural transition(s) hence, reversible in nature. Further, LdTR possesses a stable intermediate that retains 50 % structure and dimerization while the unfolded polypeptide includes FAD binding and tryptophan residues. Such intermediates (I_{U2} at 8M and I_G at 2.5M) can resume enzyme activity after removal of denaturants. However, higher concentrations of GdmCl are able to unfold this compact stable intermediate leading to complete unfolding of the protein.

Partially folded proteins may be more malleable and their intrinsic structural plasticity may enhance their responsiveness to different cellular environments. This is especially important in case of parasite proteins, like TR, which provides the main line of defense against stress conditions. Additionally, it is also involved in the pathways accounting for the disastrous phenomenon of drug resistance in *Leishmania*. Since LdTR currently represents one of the most attractive antitrypanosomal drug targets, our findings are significant as they provide intriguing insight into the mechanism/s of modulation of structure and function on exposure to ionic as well as hydrophobic moieties.

5. Conclusion

Comparative studies on the effects of urea and guanidine hydrochloride on the structural and functional properties of LdTR demonstrate that loss of activity in LdTR is not generally associated with the loss of structure. Rather the enzyme registers reversible functional changes at low concentrations of denaturants. Additionally, LdTR was found to be resistant to urea unfolding as 50 % of secondary structures and active site disulfide remain intact in urea. Further guanidine hydrochloride induces inactivation followed by the formation of molten globule intermediate species which are functionally reversible. This study for the first time reports the changes in secondary structure, FAD binding, associative behavior and presence of intermediate species, including the MG state in the unfolding pathway of trypanothione reductase from any trypanosomatid.

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Figures legends:

Fig. 1. Spectroscopic properties of native recombinant leishmanial trypanothione reductase (LdTR) under native conditions i.e. at 25°C, pH 7.5 (A) Far UV CD spectrum (B) Tryptophan fluorescence emission spectrum (C) FAD fluorescence spectrum (D) Absorption spectra of native (curve a) and NADPH reduced LdTR (curve b).

Fig. 2. Effect of increasing concentrations of urea on functional and structural properties of LdTR (A) Changes in enzyme activity of LdTR on treatment with increasing concentrations of urea as a function of enzyme concentration, (\diamond) 0.025 $\mu\text{g/mL}$, (\square) 0.05 $\mu\text{g/mL}$, (Δ) 0.1 $\mu\text{g/mL}$. Data are percentages with enzyme activity observed for LdTR in absence of urea taken as 100%. (B) Urea induced changes in secondary structure as monitored by following the changes in CD ellipticity at 222 nm from Far-UV CD curves at increasing concentrations of urea. Data are represented as the percentages of ellipticity at 222 nm, taking the value observed for native protein in absence of urea as 100 %. Inset shows the CD curves of native, 4 M and 8 M urea denatured LdTR (curve a-c) (C) Changes in tryptophan fluorescence of LdTR on treatment with increasing concentrations of urea as monitored by changes in emission maxima (squares) and fluorescence intensity at emission maxima (circles). Inset shows the fluorescence curves of native, 4 M and 8 M urea denatured LdTR (curve a-c) (D) Urea-induced changes in FAD Fluorescence intensity at 525 nm. Inset shows the fluorescence curves of native, 4 M and 8 M urea denatured LdTR (curve a-c) (E) Urea induced unfolding transitions of LdTR as obtained from CD ellipticity data (panel B), tryptophan fluorescence (panel C) and FAD fluorescence (panel D) represented by \blacksquare , \blacktriangle and \blacklozenge respectively. A linear extrapolation of the baselines in the pre- and post- transitional regions was used to determine the fraction of the folded protein within the transition region assuming a two-state mechanism of unfolding. 8 M urea denatured state was taken as completely denatured state under the present set of experimental conditions used.

Fig. 3. Urea-induced changes in LdTR conformation. (A) Dependence of ANS fluorescence in the presence of LdTR on urea concentration: fluorescence intensity (\bullet) and emission maxima (\blacksquare). Inset shows the emission curves for ANS binding at 0 M, 2 M, 4 M and 8 M (curves a-d). (B) Quenching of intrinsic fluorescence of LdTR by acrylamide at different urea concentrations 0 M (\blacksquare), 1 M (\square), 2 M (Δ), 3 M (\diamond), 4 M (\circ) and 8 M (\bullet) F_0 : fluorescence intensity in the absence of acrylamide, F : observed fluorescence intensity in presence of acrylamide (C) Thiol group titrations: \square Native state, \square NADPH-reduced state, \square 8M urea denatured state, \square 8M urea denatured state reduced with NADPH. (D) Renaturation of LdTR after treatment with various concentrations of urea. The protein was allowed to renature by diluting the denaturant to a final concentration of 10 mM and assayed for resumed enzyme activity.

Fig. 4. Dimer /monomer conversion of LdTR in presence of increasing concentrations of urea. Inset shows the SDS-PAGE gel of the cross linked protein at urea concentrations: lane1, 0M; lane2, 1M; lane3, 2M; lane 4, 3M; lane 5 4M; lane 6, 6M; lane 7, 8M. D and M represent dimer and monomer.

Fig. 5. Changes in functional and structural properties of LdTR in presence of increasing concentrations of GdmCl. (A) Changes in enzymatic activity of LdTR on incubation with increasing concentrations of GdmCl as a function of enzyme concentration, (\diamond) 0.05 $\mu\text{g/mL}$, (\square) 0.05 $\mu\text{g/mL}$, (Δ) 0.1 $\mu\text{g/mL}$. Data are represented as percentages, with enzyme activity observed for LdTR in absence of GdmCl taken as 100%. (B) Changes in CD ellipticity at 222 nm for LdTR on treatment with increasing concentrations of GdmCl. Data are represented as the percentages, with the value observed for native LdTR in absence of GdmCl taken as 100%. Inset shows the CD curves of native, 2.5 M GdmCl denatured and 6 M GdmCl denatured LdTR (curve a-c) (C) GdmCl-induced changes in tryptophan fluorescence emission of LdTR as monitored by changes in emission maxima (squares) and fluorescence intensity at emission maxima (circles). Inset shows the fluorescence curves of native, 2.5M GdmCl denatured and 6 M GdmCl denatured LdTR (curve a-c) (D) Changes in FAD Fluorescence intensity at 525 nm on treatment with increasing concentrations of GdmCl. Inset shows the fluorescence curves of native, 2.5M GdmCl denatured and 6 M GdmCl denatured LdTR (curve a-c) (E) GdmCl-induced unfolding transitions of LdTR as obtained from CD ellipticity data (panel B), tryptophan fluorescence (panel C) and FAD fluorescence (panel D) represented by \blacksquare , \blacktriangle and \blacklozenge respectively. A linear extrapolation of the baselines in the pre- and post- transitional regions was used to determine the fraction of folded protein within the transition region assuming a two-state mechanism of unfolding.

Fig. 6. Changes in protein conformation upon GdmCl denaturation. (A) ANS binding characteristics of LdTR in various GdmCl concentrations: fluorescence intensity (\bullet) and emission maxima (\blacksquare). Inset shows the emission curves for ANS binding at 0 M, 1 M, 2.5 M and 4 M (curves a-d) (B) Quenching of the intrinsic fluorescence of denatured LdTR by acrylamide at GdmCl concentrations, 0 M (\blacklozenge), 0.5 M (\blacksquare), 1 M (\blacktriangle) and 2.5 M (\bullet). F_0 : fluorescence intensity in the absence of acrylamide, F : observed fluorescence intensity in presence of acrylamide (C) Thiol group titrations: \square Native state, \blacksquare NADPH-reduced state, \square 2.5M GdmCl denatured LdTR, \square 4M GdmCl denatured LdTR, \square 4M GdmCl denatured LdTR reduced with NADPH, \square 6M GdmCl denatured LdTR. (D) Renaturation of LdTR: After treatment with various concentrations of GdmCl, the enzyme was allowed to resume its native state by diluting the denaturant to a final concentration of 2.5 mM and assayed for enzyme activity

Fig. 7. Dimer /monomer conversion of LdTR by increasing concentrations of guanidine-hydrochloride Inset shows the SDS-PAGE gel of the cross linked protein at the GdmCl concentrations: lane1, 0M; lane2, 1.5M; lane3, 2.5M; lane 4, 3M; lane 5, 3.5M; lane 6, 4M. D and M represent dimer and monomer.

Fig. 8. Reactivation and unfolding of 8 M urea denatured intermediate by increasing concentrations of GdmCl. (A) Reactivation profile of LdTR denatured with 8 M urea (bar 1), 8 M urea + 100 mM GdmCl (bar 2), 8 M urea + 250 mM GdmCl (bar 3), 8 M urea + 500 mM GdmCl (bar 4), 8 M urea + 750 mM GdmCl (bar 5). (B) CD spectra of native LdTR (curve a) and LdTR denatured with 8 M urea (curve b), 8 M urea + 500 mM GdmCl (curve c) and 8 M urea + 1 M GdmCl (curve d).

Fig. 9. Diagrammatic representation of the differential modes of unfolding of LdTR by GdmCl and urea.

Table 1

Calculated secondary structure fractions in LdTR

Method	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	NRMSD
CDSSTR	0.15	0.11	0.16	0.10	0.20	0.28	0.018
CONTIN	0.120	0.107	0.121	0.083	0.234	0.335	0.022
SELCON3	0.141	0.114	0.112	0.083	0.229	0.310	0.063

Table 2

Values of Stern Volmer quenching constants

Urea denaturation		GdmCl Denaturation	
Concentration	$K_{sv} [M^{-1}]$	Concentration	$K_{sv} [M^{-1}]$
0 M	3.9 ± 0.18	0 M	3.9 ± 0.18
2 M	3.82 ± 0.5	0.5 M	6.1 ± 0.82
4 M	4.6 ± 0.51	1 M	6.34 ± 0.73
8 M	7.32 ± 0.46	2.5 M	8 ± 0.52

Figure 1

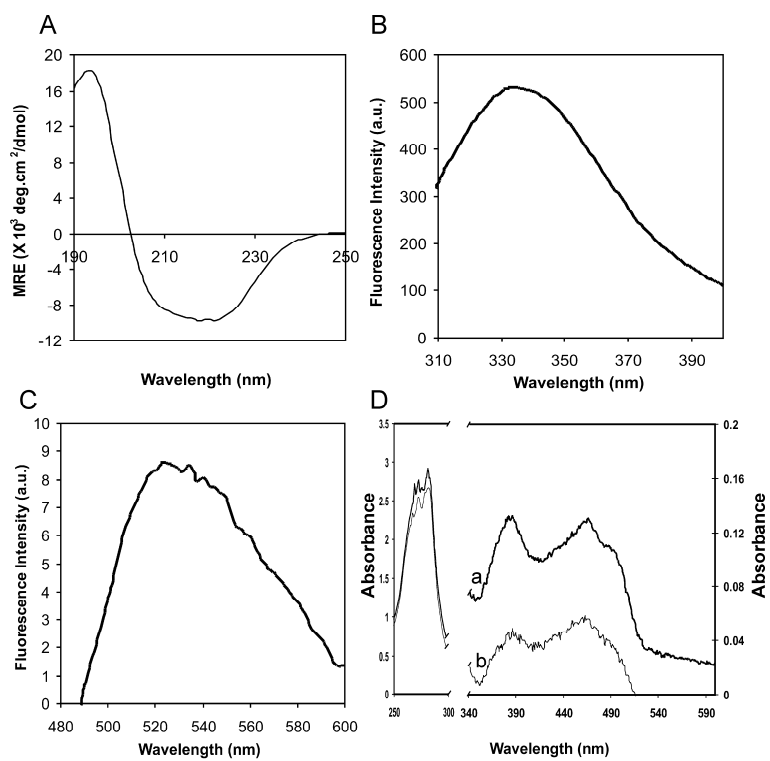


Figure 2

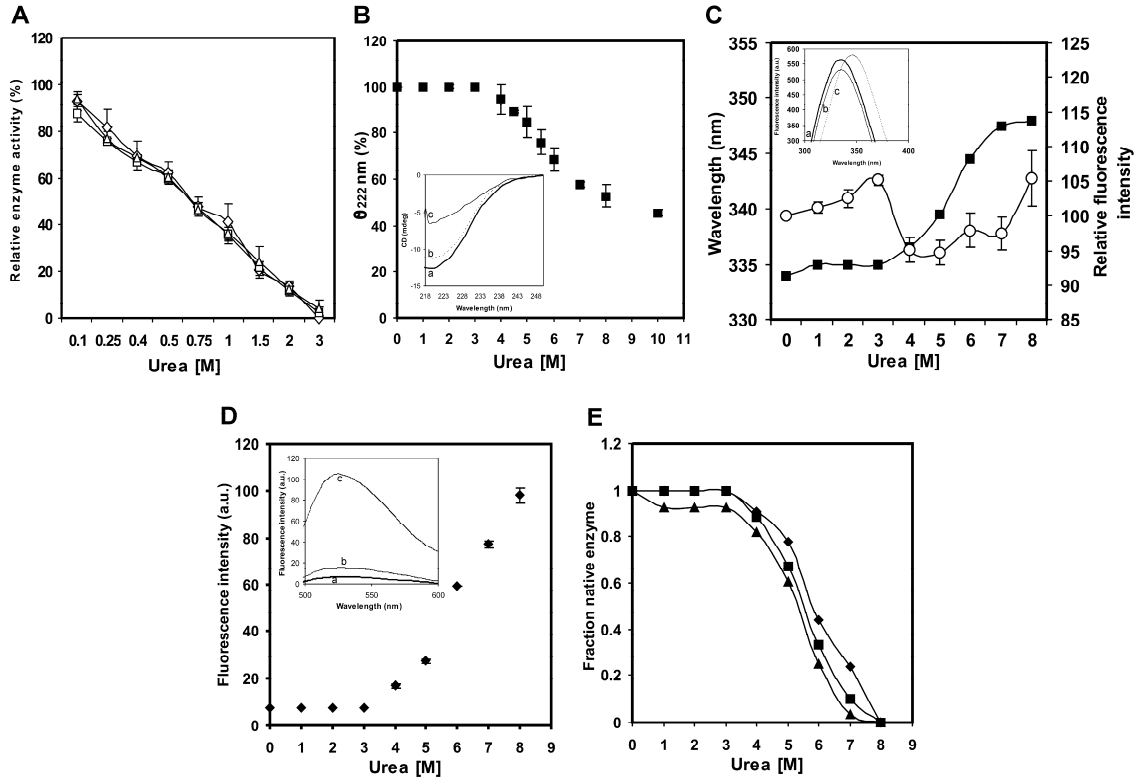


Figure 3

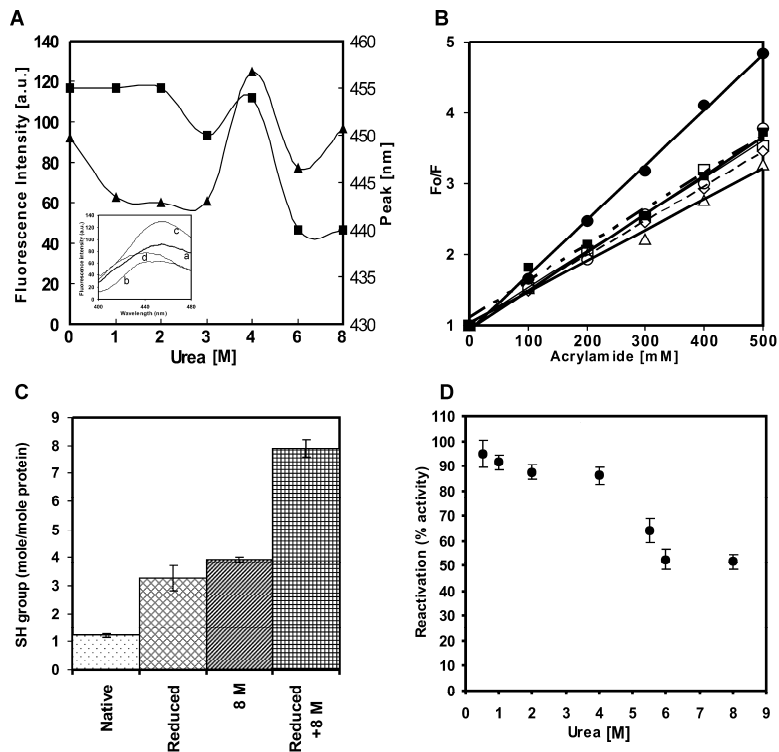


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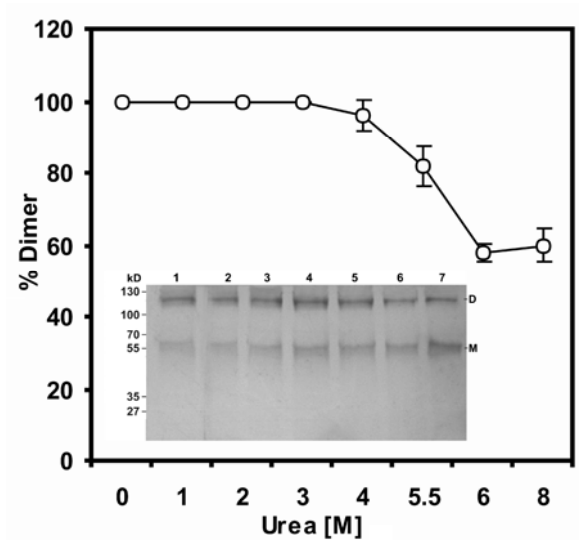


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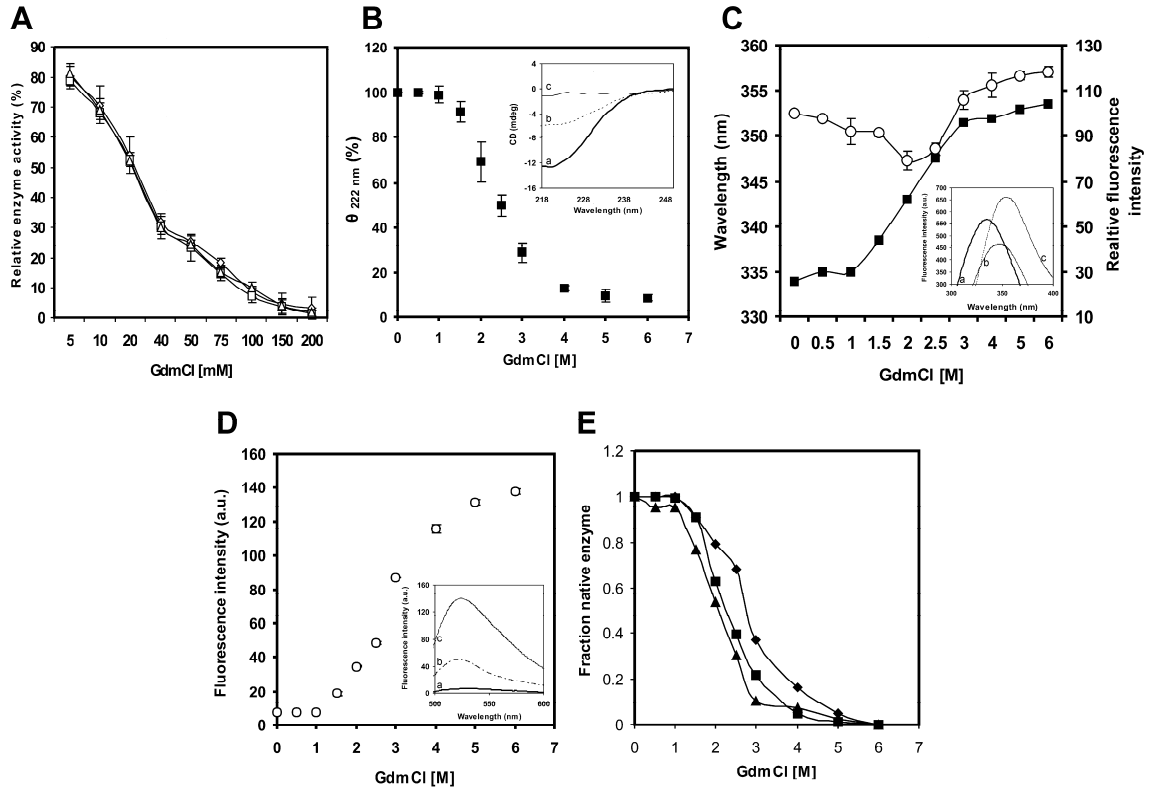


Figure 6

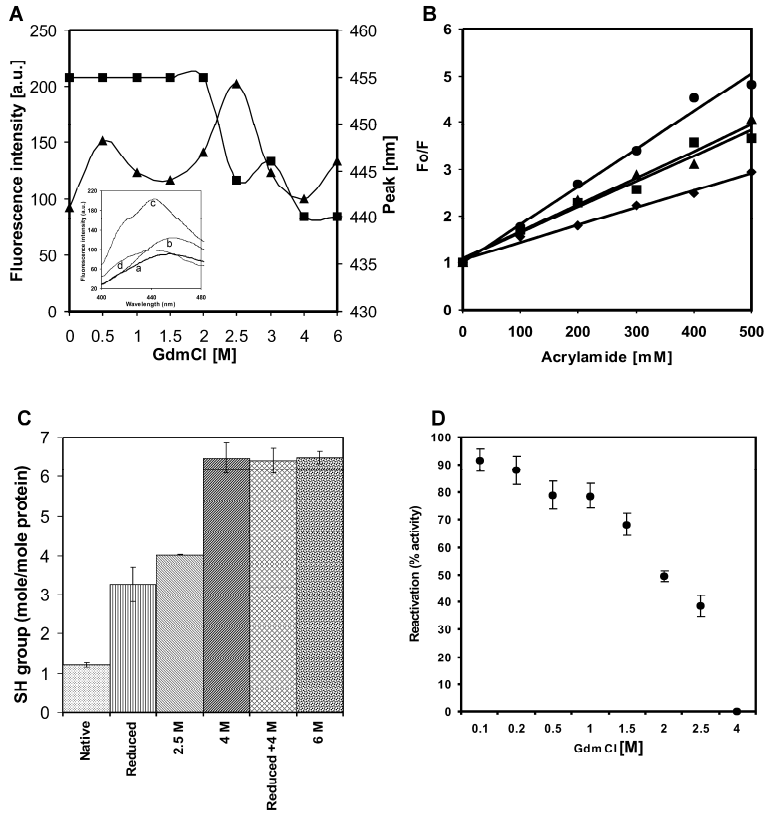


Figure 7

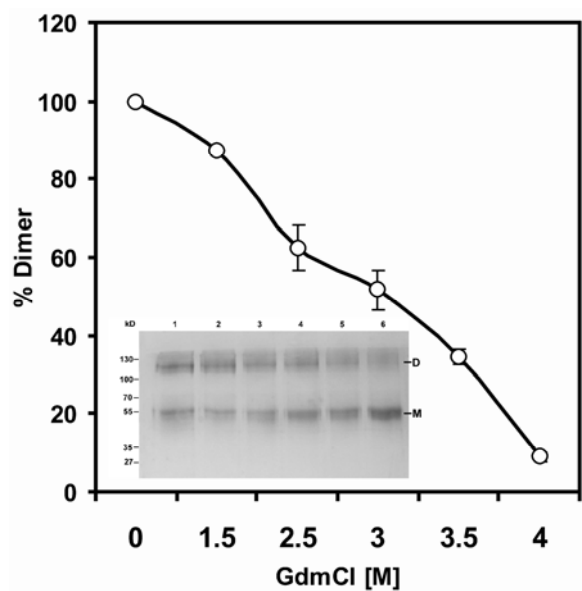


Figure 8

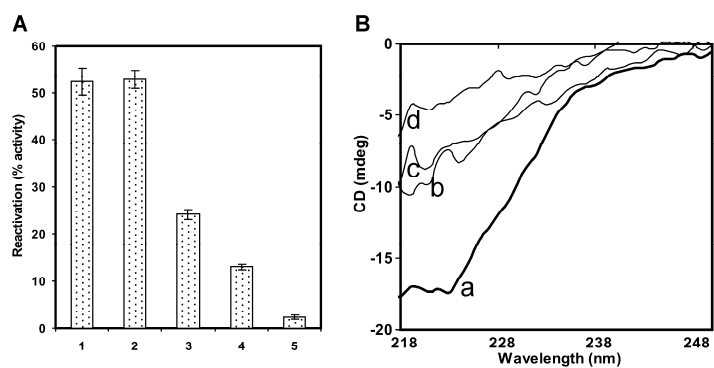


Figure 9

