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Biophysical and Folding Parameters of Trypanothione Reductase from *Leishmania infantum*

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Abstract Out of various tropical diseases caused by trypanosomatids, leishmaniasis is a life-threatening disease caused by the leishmania parasite. We are targeting the thiol metabolic pathway of the parasite for drug development, and trypanothione reductase (TryR) is a key enzyme of this pathway. It is important to gather significant knowledge about biophysical and intrinsic properties of this enzyme which will be helpful in better understanding of this drug-target enzyme. We report here the modulation of activity and stability of TryR from *Leishmania infantum* in the presence of various denaturants and pHs. The enzyme is quite stable under high concentration of denaturants and showed better stability compared to TryR of *Leishmania donovani*, whose sequence differs at only on position (Ala363→Gly). Structural basis of the destabilizing effects is discussed.

Keywords Protein stability · Protein · Folding · Leishmaniasis · Drug-target enzyme

Introduction

Trypanothione has two glutathionyl moieties linked together by spermidine linkage [1]. Tryanothione reductase (TryR) is involved in the unique thiol metabolism of trypanosomatids such as *Leishmania* and *Trypanosoma* and keeps trypanothione in reduced form. These trypanosomatids are causative agents of several medicinally challenged infectious diseases such as African sleeping sickness (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*) and visceral leishmaniasis (*Leishmania donovani* and *Leishmania infantum*) [2]. According to current WHO statistics, 12 million people living in 88 countries are infected with leishmaniasis with an annual increase of 1.5–2 million new cases [3]. This disease is still common in rural areas and is endemic in Central and South American countries [4]. The treatment of the disease is mainly based upon chemotherapy and most of the drugs are toxic, of high cost, and low efficacy [5–7].

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These protozoan parasites have an entirely different thiol metabolism to tackle with oxidative stress compared to their insect (phlebotomine sand fly) and mammalian hosts. They possess N^1,N^8-bis (glutathionyl) spermidine (trypanothione) which is kept reduced by TryR. TryR transfers reducing equivalents from NADPH to oxidized trypanothione via FAD [8, 9] and finally reduced trypanothione helps in removal of reactive oxygen species and formation of DNA precursors [10]. TryR is a member of NADPH-dependent flavoprotein oxidoreductase and functional analogue of glutathione reductase (GR) which is present in mammalian host [11]. GR and TryR are highly specific for their substrates, i.e., glutathione and trypanothione, respectively.

The activity of several proteins is altered in presence of denaturants like urea and GuHCl due to modulation of their conformations [12]. In the last few years, focus has been directed towards studying the mechanism denaturation/renaturation of proteins to get insights into folding and stability [13]. In view of its important role in oxidative stress removal and unique nature, TryR becomes an interesting target for chemotherapy of leishmaniasis. We have attempted here to study biophysical properties of TryR to evaluate its stability and activity parameters in presence of different denaturants such as urea, guanidine hydrochloride (GuHCl) and pH which can provide information about conformational stability of proteins [14]. Unfolding results are reported on TryR from a different species of leishmania, i.e., *L. donovani* [15]. Interestingly, we found that TryR of *L. infantum* is more stable and active in extreme conditions compared to that of *L. donovani*. Sequence analysis of these proteins shows that TryR of *L. donovani* differs from the enzyme form *L. infantum* at only on position (Ala363→Gly). The structural basis of the stability effect was interpreted. Moreover, differences in folding/unfolding patterns of TryR from these two species are also discussed.

Materials and Methods

Trypanothione was purchased from BACHEM, Switzerland. TryR gene inserted in the *Nde*I–*Hin*dIII unique sites of pET28b Novagen, in frame with N-terminal His-tag was generously donated by Dr. Andrea Ilari and Gianni Colotti, Università "La Sapienza" Rome, Italy. Thrombin, 8-anilinonaphthalene 1-sulfonic acid (ANS), urea, and guanidine hydrochloride (GuHCl) were of analytical grade.

Enzyme Expression, Purification and Activity Assays Recombinant *L. infantum* TryR was expressed in *Escherichia coli*, purified using Ni-affinity chromatography, and 6× His-tag was removed using the method reported earlier [16]. The enzyme was stored at 4 °C in phosphate buffer at pH 7.5. Microtiter plate assay was carried out on Tecan spectrophotometer using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as an indicator for reduced thiol groups [17]. The final assay mixture (250 μl) contained TryR (1 m unit), 40 mM HEPES (pH 7.5), 15 mM NADPH, 1 mM EDTA, 25 μM DTNB, and different concentrations of trypanothione $\{T(S)_2\}$ ranging from 0.5 to 50 μM. Enzyme mixture was pre-incubated with NADPH for 5 min at 26 °C. The reaction was started by the addition of substrate. Enzyme activity was monitored at 412 nm to detect the conversion of DTNB into yellow-colored TNB. Lineweaver–Burk plot (double reciprocal plot) was generated for calculation of K_m and V_{max}.

Measurement of Fluorescence All fluorescence spectroscopic measurements were done using a Varian fluorescence spectrophotometer and 10-mm path-length quartz cuvette. FAD fluorescence was monitored by exciting at 460 nm and emission recorded between 480 and 600 nm. Tryptophan fluorescence was monitored by exciting at 290 nm and emission recorded

between 300 and 400 nm [13]. Both the changes in fluorescence intensity and wavelength maxima were plotted against denaturant concentration to reveal unfolding pattern.

ANS Binding Experiments ANS binding experiment was performed by incubation of native and denaturant-treated samples with 50 μM ANS for 30 min. The ANS fluorescence was monitored by exciting the sample at 380 nm and recoding the emission between 400 and 600 nm [18].

Estimation of Denaturation by Urea and GuHCl TryR (7 μM) enzyme was incubated in the presence of different concentrations of urea and GuHCl (ranging from 0.2 to 8 M of urea and 0.2 to 6 M of GuHCl) overnight at room temperature. After the incubation, fluorescence spectra for FAD, tryptophan, and ANS were measured as previously discussed. Enzyme inactivation by urea and GuHCl was studied by incubating the samples for 1 and 24 h. We have chosen longer time of incubation as there was no significant effect on enzyme activity after incubation for 1 h.

Circular Dichroism Measurements CD measurements were performed using Jasco J715 spectropolarimeter equipped with constant temperature cell holder. CD spectra were collected in the range of 200–250 nm using a quartz cuvette of 1 mm path length and scan speed of 40 nm/min. Final protein concentration was kept at 5 μM. Protein secondary structure prediction was done using K2D2 online server.

Estimation of Sulfhydryl Groups in Native and Denatured TryR DTNB (Ellman's reagent) works as an indicator for the reduced thiol groups present in the protein. It is converted to colored TNB molecule by a reduced thiol group which gives a yellow color and absorbance can be measured at 412 nm. Enzyme mixture, containing 7 μM native TryR, NADPH-reduced TryR, urea denatured TryR and urea-denatured-cum-NADPH-reduced TryR, 1 mM EDTA in 100 mM Tris buffer (pH=8.2), was incubated with 100 μM DTNB in four different microtiter wells and absorbance was monitored at 412 nm. Three hundred micromolar NADPH was used for reduction of TryR enzyme.

Estimation of Renaturation of TryR To estimate the renaturation capability of TryR, firstly, enzyme was denatured in presence of different urea and GuHCl concentrations for 24 h and then the mixture was dialyzed extensively to remove the denaturant. The enzyme activity was measured after incubation of 1 h at room temperature. Fluorescence spectra were also similar to the native TryR.

Estimation of Effect of pH on TryR Stability and Activity Native TryR was incubated in buffers of different pH ranges (0.5 to 12.5) namely 50 mM KCl–HCl (pH 1.0–1.5), 50 mM glycine–HCl (pH 2.0–3.5), 50 mM sodium acetate (pH 4.0–5.5), 50 mM sodium phosphate buffer (pH 6.0–7.5), 50 mM Tris–HCl (pH 8.0–10.5), and 50 mM glycine–NaOH (pH 11–12.5), respectively, for 24 h at room temperature followed by estimation of enzyme activity, tryptophan, and FAD fluorescence.

Results

Structural Features of TryR Tryptophan fluorescence spectrum of TryR shows a typical peak at 334 nm, indicating a non-polar environment of tryptophan residue inside the protein

(Fig. 1a). Native TryR, when excited at 460 nm and emission recorded between 480 and 600 nm, gave emission maxima at 525 nm which is the characteristic of FAD group present

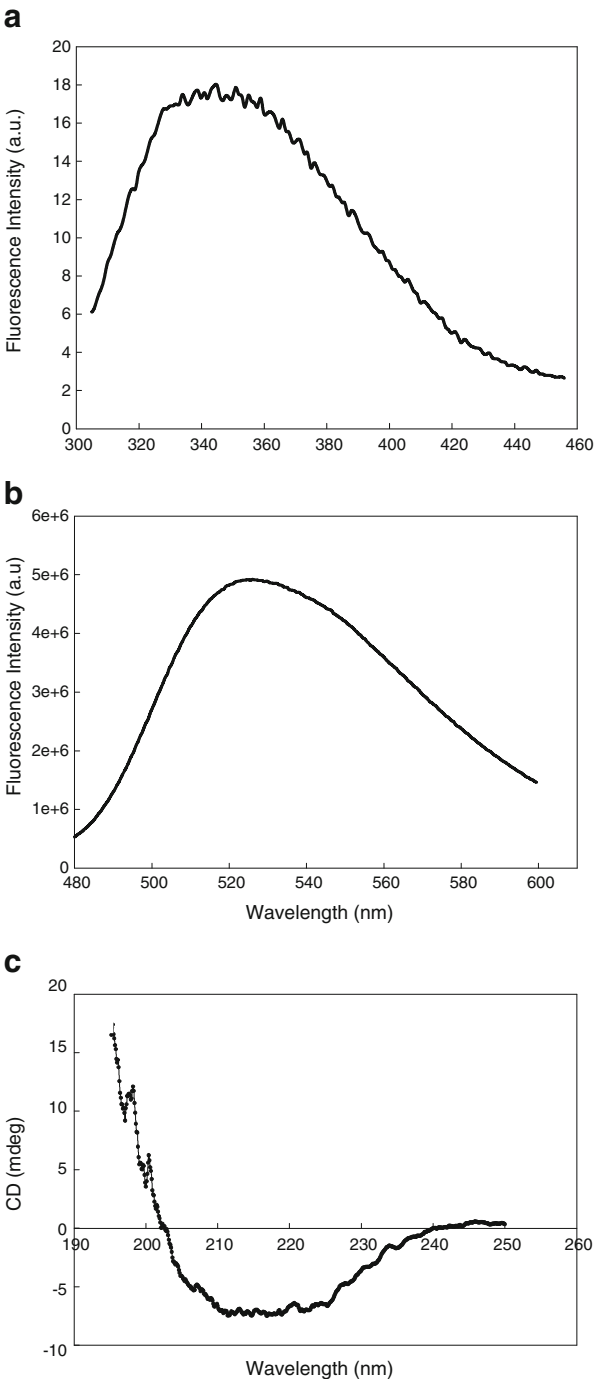

Fig. 1 Spectroscopic characterization of *Leishmania infantum* TryR. **a** Tryptophan fluorescence **b** FAD fluorescence, **c** Far UV CD spectrum

in the protein (Fig. 1b). Far-UV circular dichroism spectra of the native protein shows a typical α/β helix pattern with 57.3±0.4% α helix and 6.1±0.2% β strands as calculated (Fig. 1c).

Urea-induced Unfolding of TryR Urea-induced changes are given in Fig. 2. The unfolding transitions by urea show biphasic nature (Fig. 2a and c). The tryptophan fluorescence emission spectra of TryR shows emission maxima at 334 nm for native state which shifts to 358 nm upon denaturation by 8 M urea (Fig. 2b). Fluorescence intensity was also increased in the presence of urea compared to native state. The emission fluorescence maxima around 334 nm is the characteristic of tryptophan residues buried inside the hydrophobic core of TryR whereas emission maxima above 350 nm shows that all the tryptophan residues are exposed to polar environment after denaturation. The FAD fluorescence intensity does not change up to 3 M urea and then increases linearly up to saturation after 6 M urea (Fig. 2c). The 3 M concentration of urea is insufficient for unfolding the protein so that at the above concentration, the FAD group is not exposed to aqueous environment. Beyond this concentration of denaturant/unfolding agent, the protein unfolds and the FAD group now comes in contact with aqueous environment and hence a linear increase in FAD fluorescence intensity with concentration was observed. FAD is tightly bound to N-terminal domain through hydrogen bonds [2]. ANS is an extrinsic fluorescence dye which is widely used for probing conformational changes in proteins. ANS non-covalently binds with the hydrophobic core of proteins and has low fluorescence in a polar environment. It is widely used in protein folding/unfolding experiments and biological membranes [19]. We found a sharp decrease in emission maxima and enhanced ANS fluorescence at ~4 M urea concentration. This pattern indicates the formation of a molten globule-like intermediate state at this concentration (Fig. 2d). This is further substantiated by biphasic unfolding transition of urea (Fig. 2a and c). Through our inactivation studies, we found that TryR retains its activity up to longer time and denaturation is reversible. TryR of *L. infantum* retains 76.02 ±2.9% and 41.17±2.4% activity in the presence of 2 M urea after 1 and 24 h, respectively (Fig. 2e). This result indicates that TryR from *L. infantum* is active for a longer time compared to the reported complete inactivation of TryR from *L. donovani* only after 1 h [15]. Denaturation of TryR by urea is partially reversible (Fig. 2f). It regains activity up to 54.91± 2.2% after denaturation in 8 M urea. We have estimated the amount exposure of sulfhydryl groups (accessible –SH group) at different concentrations of denaturant as a parameter of unfolding (Fig. 2g). Native TryR has 0.8±0.23 mol/mol of protein sulfhydryl content whereas TryR reduced by NADPH, urea denatured TryR, and urea-denatured-cum-NADPH-reduced TryR have 4.6±0.87, 4.8±0.83, and 12.34±1.23 mol/mol of protein sulfhydryl content, respectively.

GuHCl-induced Unfolding of TryR GuHCl-induced changes are given in Fig. 3. The unfolding transitions via GuHCl also show biphasic nature (Fig. 3a, c). Additionally, a red shift in tryptophan fluorescence wavelength maxima from 334 to 386 nm with increase in fluorescence intensity was observed (Fig. 3a). When FAD fluorescence intensity was used as probe, no structural change has been seen up to 2 M GuHCl and then a cooperative unfolding was seen. It shows that exposure of FAD to polar environment starts at 2 M and it is completed at 3.2 M GuHCl. The protein shows a high ANS binding experiments at low concentration (~1 M) of GuHCl concentration (Fig. 3d). Strong ANS binding at low GuHCl concentration shows formation of intermediate state at lower GuHCl concentration. Interestingly, TryR from *L. infantum* is active even after 24 h of GuHCl treatment and contains 74.67±2.2% and 13.33±2.9% activity in 2 M GuHCl after 1 and 24 h, respectively

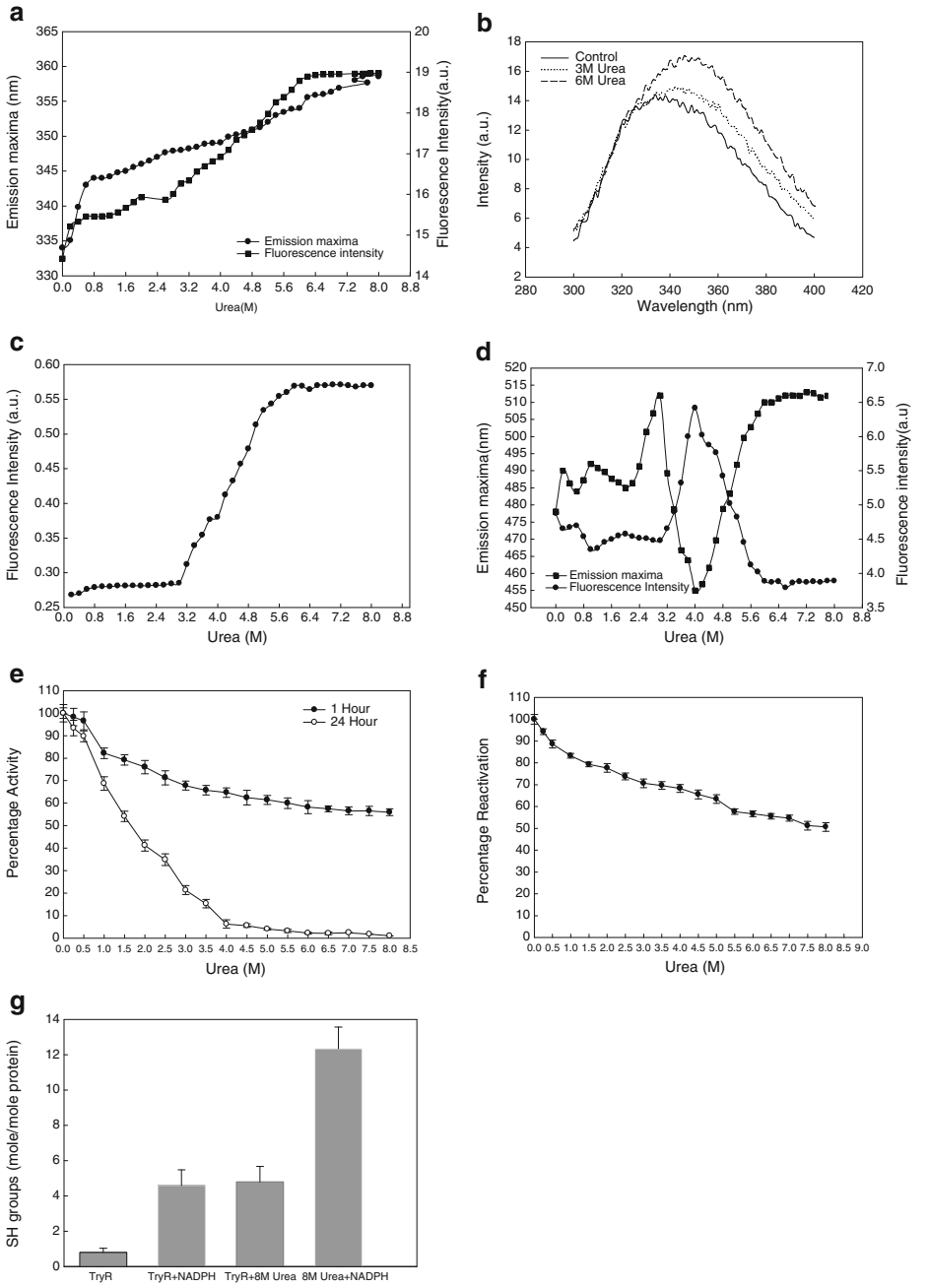

Fig. 2 **a** Urea-induced changes as followed by tryptophan emission maxima and fluorescence intensity at emission maxima. **b** Tryptophan fluorescence at 0, 3, and 6 M urea concentration. **c** Urea-induced changes in FAD fluorescence intensity at 525 nm. **d** Urea-induced changes in ANS-fluorescence intensity and emission maxima. **e** Enzyme inactivation profile with increasing concentration of urea. **f** Reactivation of denatured TryR with urea. **g** Sulfhydryl group estimation in native TryR, NADPH-reduced TryR, 8-M-urea-denatured TryR and NADPH-reduced-cum-8-M-urea-denatured TryR

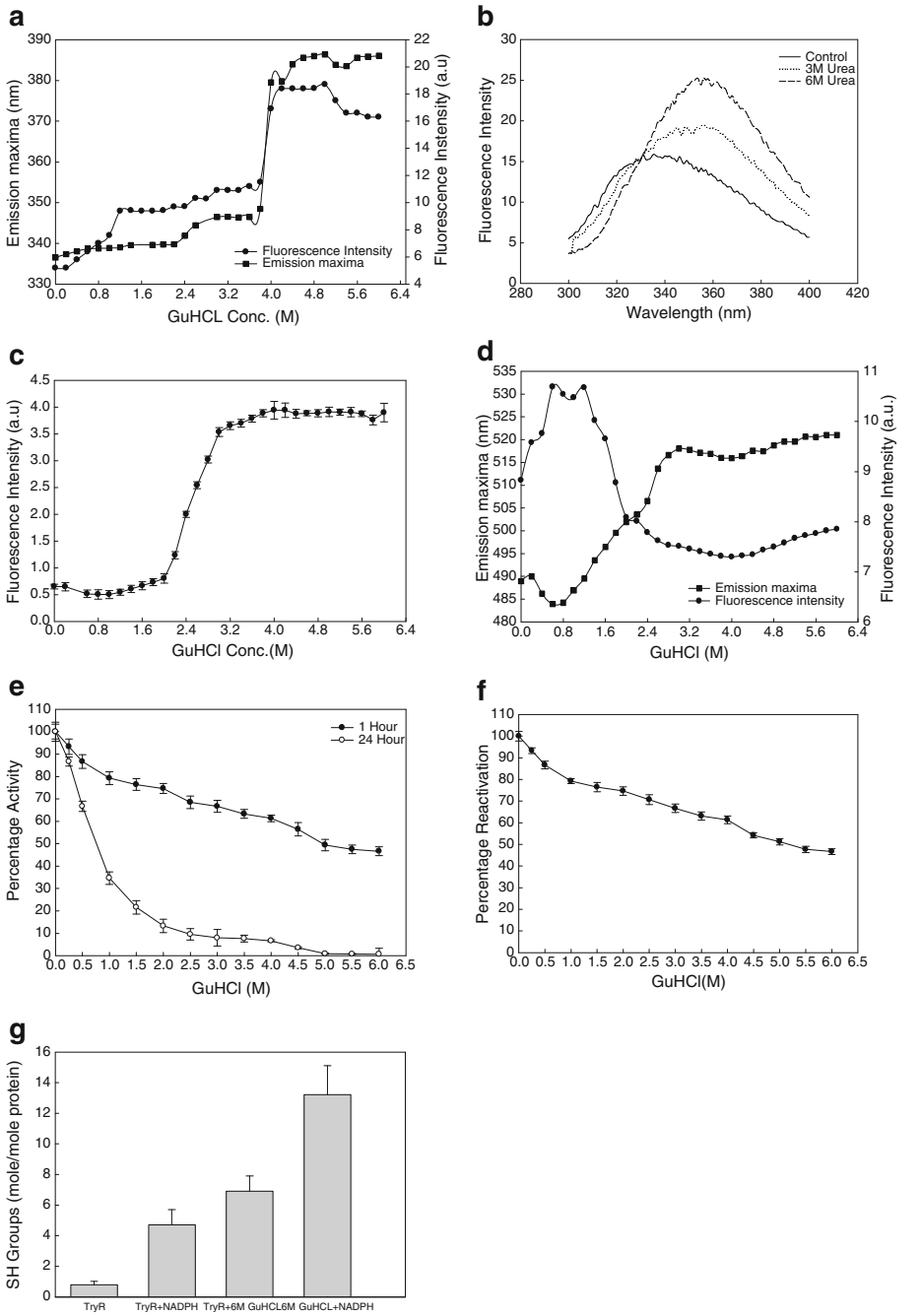

Fig. 3 **a** GuHCl-induced changes as followed by tryptophan emission maxima and fluorescence intensity at emission maxima. **b** Tryptophan fluorescence at 0, 3, and 6 M GuHCl concentration **c** GuHCl-induced changes in FAD fluorescence intensity at 525 nm. **d** GuHCl-induced changes in ANS-fluorescence intensity and emission maxima. **e** Enzyme inactivation profile with increasing concentration of GuHCl. **f** Reactivation of denatured TryR with GuHCl. **g** Sulfhydryl group estimation in native TryR, NADPH-reduced TryR, 8-M-urea-denatured TryR and NADPH-reduced-cum-6-M-GuHCl-denatured TryR

(Fig. 3e). Further, TryR inactivation is found to be reversible when denatured by GuHCl whereas TryR activity from *L. donovani* is reported to be irreversible after denaturation with GuHCl (Fig. 3f). Once denatured with 6 M GuHCl, the protein regains 45.73±2.11% once GuHCl is dialyzed. Additionally, accessibility of sulfhydryl groups was also used as probe for unfolding studies (Fig. 3g).

pH-induced Changes pH-induced changes of TryR are shown in Fig. 4. Change in tryptophan and FAD fluorescence as function of pH indicates distortion in the protein structure which leads to unfolding and inactivation of TryR. Moreover, our results show that TryR is active in the pH range of 6.0–11.0, showing maximum activity at pH 7.5.

Discussion

The biological function of a protein depends upon the correct folding pattern of the protein. Denaturants like urea and GuHCl affect intrinsic properties of protein such as secondary structure, function, and stability [20, 21]. Equilibrium unfolding of TryR indicates that this dimeric protein is quite stable at high concentrations of denaturants and extreme pH range and unfolding pathway involves formation of intermediate states. In our current study, we found TryR of *L. infantum* more stable and active in denaturing environment of urea and GuHCl as compared to TryR of *L. donovani*.

The main aim of our current study was to explore the differences in activity and stability of TryR from two Leishmania species i.e. *L. infantum* and *L. donovani*. We have done experimental analysis on TryR of *L infantum* (Li-TryR) whereas folding and stability parameters of TryR of *L. donovani* (Ld-TryR) are already reported [15]. Interestingly, we found that Li-TryR is less prone to unfolding and inactivation compared to Ld-TryR. Li-TryR retains about 70% activity after 1 h and about 20% activity after 24 h in presence of 3 M urea concentration while the activity of Ld-TryR is completely lost only after 1 h of 3 M urea treatment. Li-TryR does not lose more than 40% activity even at 8 M urea concentration after 1 h. There is a sharp decrease in ANS emission maxima at 2 M concentration with Ld-TryR while the same is at 3 M urea concentration with Li-TryR, i.e., Ld-TryR tends towards the intermediate formation step at lower concentration of urea compared to Li-TryR. Tryptophan and FAD fluorescence patterns also differ between these two species as there is sharp increase in emission maxima of both tryptophan and FAD fluorescence in Ld-TryR while their transitions are non-cooperative in case of Li-TryR which shows population of folding intermediate. Careful inspection of urea and GuHCl unfolding by tryptophan shows three transitions while unfolding by FAD fluorescence shows cooperative transition with higher transition mid-point (although little biphasic nature is visible in urea unfolding). Thus, FAD binding N-terminal domain seems to have better stability and unfolds late. During denaturation with GuHCl, Ld-TryR shows comparatively very high instability and inactivation as only 200 mM GuHCl inactivates it completely after 1 h; whereas Li-TryR retains 95% activity after 1 h and 86% activity after 24 h in 500 mM GuHCl. Li-TryR does not lose more than 50% activity even at very high concentration of GuHCl, i.e., 6 M after 1 h. FAD fluorescence intensity is increased after 1 M of GuHCl in Ld-TryR while it is increased after 2 M GuHCl in Li-TryR, i.e., FAD is exposed to polar solvent at higher concentration of GuHCl in Li-TryR. Also, a tryptophan fluorescence emission maximum sharply

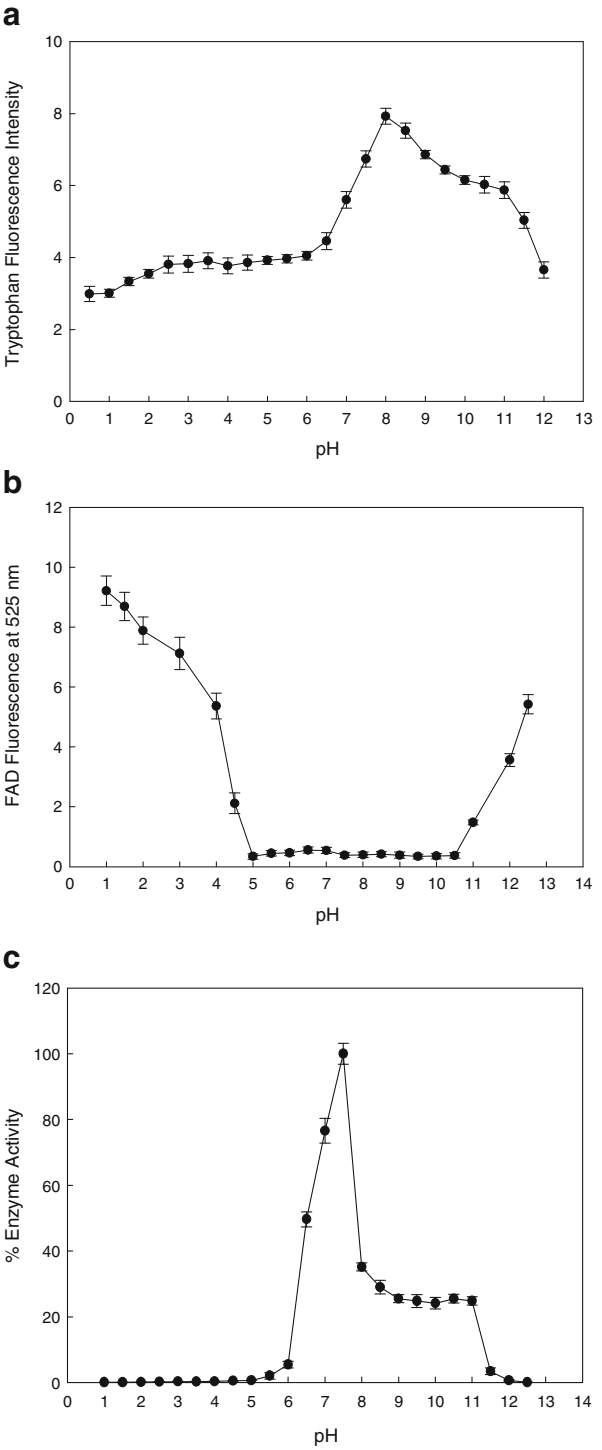

Fig 4 Effect of pH on properties of TryR. **a** Modulation of tryptophan fluorescence, **b** modulation of FAD fluorescence and **c** effect on enzymatic activity. All data were collected in the pH range of 0.5–12.5

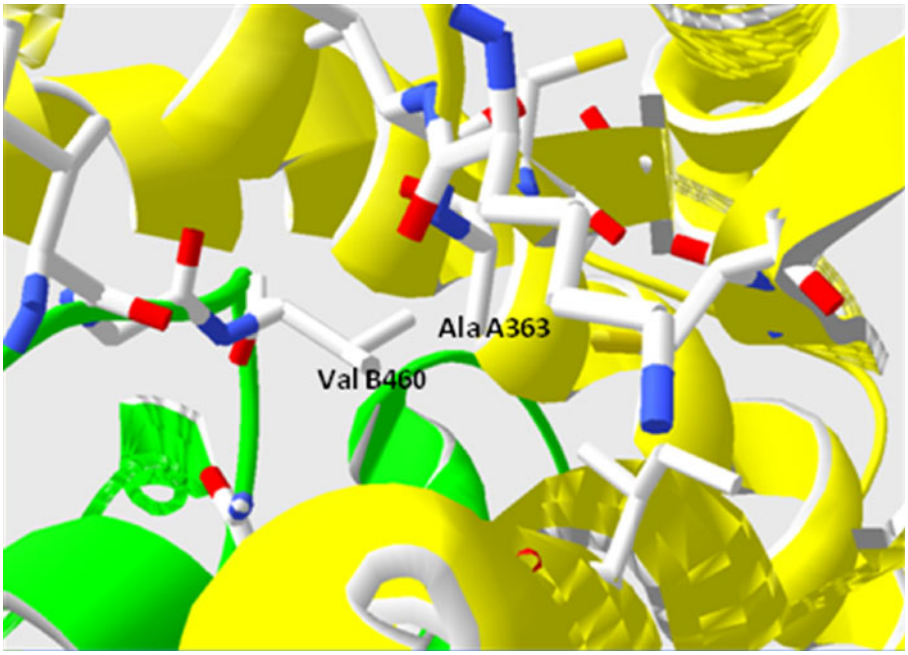

Fig. 5 Figure showing packing of Ala A363 against Val B460 at dimeric interface of TryR for *Leishmania infantum* (PDB ID: 2JK6)

increases just after 1 M GuHCl in Ld-TryR while it is almost stable up to 3.6 M GuHCl and then increases. Thus, all these experimental results lead us to conclude that Li-TryR is more stable and active in higher concentration of urea and GuHCl compared to Ld-TryR.

To understand the structural basis of better stability of TryR from *L. infantum*, the crystal structure of the enzyme was analyzed. The only difference between the proteins from these two *Leishmania* species is that Ala363 in *L. infantum* is replaced by Gly residue in *L. donovani*. Position 363 in the crystal structure of the protein (PDB ID: 2JK6) appears to be part of an interface with molecule B (Fig. 5). In particular, Val B460 may pack against Ala A363 via van der Waals interaction. Thus, Gly substitution in case of TryR from *L. donovani*, would disrupt this van der Waals packing (i.e., leaving a hole). As TryR is a dimer in solution, a part of the overall stabilization energy is associated with the dimerization. Thus, the Gly mutation may destabilize the overall dimer due to interface disruption which leads to overall loss of stability. In other words, Ala substitution at position 363 in case of *L. infantum* contributes to improved stability due to better packing at the dimer interface. Additionally, Gly substitution increases the entropy of the denatured state (i.e., stabilizes it) but often have little effect on the entropy of the native state. Thus, the effect is destabilization due to unfolded state considerations. Gly is typically utilized in positions where the side chain Cβ is eclipsed (i.e., strained) with local main chain atoms. This situation arises in turn, where the main chain makes a substantial change in direction. Thus, Gly is found at certain turn positions (and stabilizes the turn by eliminating the bad contact with side chain Cβ). In this case, the secondary structure is not a turn but a β-strand. Ala is not the greatest residue for stabilizing β-strand, but Gly would be worse (due to the above-mentioned entropy considerations and loss of van der Waals contacts with the main chain).

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