JPP JOURNAL OF PROTEINS AND PROTEOMICS
6(2), 2015, pp. 153-158

Research Article

THE N-TERMINAL PORTION OF THE GLUTAREDOXIN LIKE PROTEIN 1 OF *PLASMODIUM FALCIPARUM* DOES NOT CONTRIBUTE TO THE STABILITY OF THE ENZYME

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Abstract: Maintenance of intracellular disulfide equilibrium is necessary for normal functioning of proteins. In most organisms the redox status of the cell is maintained by the thioredoxin and glutathione systems. Glutaredoxins (Grxs) are the key redox active protein of the glutathione system. The monothiol Grxs, also known as Grx-like proteins (GLPs), are mechanistically and functionally heterogeneous group of the classical dithiol Grxs. The GLP1 of *Plasmodium falciparum* has a flexible N-terminal portion that is susceptible to proteolytic cleavage. Earlier, we reported that this extension has no role in the modulation of functional behavior of PfGLP1. Using far-UV CD and fluorescence spectroscopy, we hereby demonstrate that this flexible N-terminal region does not have any role in maintaining the stability of PfGLP1. The free energy of stabilization of full-length and N-terminal deleted fragments was calculated using GdnHCl- and urea-induced denaturation curves. The results showed that the ΔG_D^{H2O} values for both the proteins were comparable. We suggest that this extension might be an evolutionary rudimentary region of the protein.

Keywords: Redox, thioredoxin, glutaredoxin, glutathione, monothiol, oxidative stress, stability, unfolding

Introduction

Malaria is the root cause of more than two million deaths per year worldwide and thus the development of novel antimalarial drugs is important (Greenwood and Mutabingwa, 2002; Olliaro, 2001). The malaria parasite *Plasmodium falciparum* is exposed to high fluxes of reactive oxygen species as the parasite multiplies in an environment of high oxygen tension. Therefore, proteins involved in antioxidant defense and redox metabolism are promising targets for antimalarial drug development (Becker *et al.*, 2000; Davioud-Charvet *et al.*, 2001; Krauth-Siegel and Coombs, 1999). In most organisms intracellular redox milieu is maintained by two parallel systems, namely thioredoxin and glutathione system. Thioredoxin system consists of thioredoxin, thioredoxin reductase and NADPH, while glutathione system consists of glutaredoxin, glutathione reductase and glutathione. Glutaredoxins are small proteins that are ubiquitously conserved throughout evolution. As thiol dependent oxidoreductases they maintain the thiol/disulphide equilibrium of the cellular proteins through the transfer of electrons from glutathione to inter- or intra- molecular disulphides (Aslund *et al.*, 1996). Glutaredoxins belongs to the thioredoxin family of proteins (Arner and Holmgren, 2000; Ferrari and Soling, 1999; Holmgren, 2000; Martin, 1995; Tripathi *et al.*, 2010; Yogavel *et al.*, 2014). The members of this family contain a highly conserved thioredoxin fold, which consists of a central five-stranded β-sheet flanked by four α-helices (Martin, 1995; Yogavel *et al.*, 2014).

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Received: October 17, 2014
Accepted: March 13, 2015
Published: March 16, 2015

Over the last few years, many proteins with quite similar sequences to classical glutaredoxins have been identified and named as glutaredoxin like proteins (GLPs). GLPs are mechanistically and functionally heterogenous group with only few similarities to canonical glutaredoxins (Deponte *et al.*, 2005; Rahlfs *et al.*, 2001). The classical glutaredoxins have an active site with two conserved cysteine residues (-CXXC-) while these GLPs contain a serine residue replacing the second cysteine in the active site and thus are also called as monothiol glutaredoxins. (-CXXS-) (Belli *et al.*, 2002; Rahlfs *et al.*, 2001; Rodriguez-Manzaneque *et al.*, 2002). In *Saccharomyces cereviseae* three monothiol glutaredoxins are found- Grx3, Grx4 and Grx5 (Herrero *et al.*, 2010). The absence of any of these proteins leads to the decrease in cellular glutaredoxin activity, even though they contain -CXXS- motif at the amino terminal region instead of the conserved active site -CXXC- residues of dithiolic glutaredoxins (Rodriguez-Manzaneque *et al.*, 1999). *Plasmodium falciparum* encodes three GLPs (PfGLP1, GLP2 and GLP3) (Deponte *et al.*, 2005; Rahlfs *et al.*, 2001). Interestingly PfGLP1 shows around 41% sequence identity with Grx5 of *S. cereviseae* (Rahlfs *et al.*, 2001), which in absence, causes several growth defects in yeast and is essential for its proper viability (Ojeda *et al.*, 2006; Pujol-Carrion et al., 2006; Pujol-Carrion and de la Torre-Ruiz, 2010; Rodriguez-Manzaneque *et al.*, 1999). In analogy to yeast, one might hypothesize an important role of GLP1 in *Plasmodium falciparum*. In contrast to other glutaredoxins, PfGLP1 lacks activity in the Glutathione: HEDS Transhydrogenase assay, while it shows slight dithiol reducing activity in the insulin assay in the presence of dithiothreitol (Rahlfs *et al.*, 2001). PfGLP1 has been included in the PICOT (for protein kinase C-interacting cousin of thioredoxin) homology domain containing proteins (Rahlfs *et al.*, 2001), alongwith the yeast Grx3, 4 and 5 (Isakov et al., 2000; Witte *et al.*, 2000), and *Arabidopsis* CXP1 and CXP2 proteins (Cheng and Hirschi, 2003).

Some time back we reported that the flexible N-terminal extension of PfGLP1 has no role in the modulation of the functional activity of the enzyme (Tripathi *et al.*, 2008). In the present study, we evaluate if the N-terminal portion has any role in maintaining the stability of PfGLP1. Our present data suggest that the non-functional N-terminal portion of the PfGLP1 has no role in contributing to the stability of the enzyme.

Materials and Methods

All chemicals used were of the highest purity and were obtained from Sigma Chemical Company, St. Louis, USA or Sisco Research Laboratories, Mumbai, India. Bacterial culture media was purchased from Himedia Laboratories, Mumbai, India.

Preparation of PfGLP1 and the fragment Thr^{10} to Ile^{127} of PfGLP1

Cloning, overexpression and purification of PfGLP1 was carried out as described earlier (Rahlfs *et al.*, 2001; Tripathi *et al.*, 2008). Limited proteolysis was performed as per the protocol of Tripathi et.al (Tripathi *et al.*, 2008).

GdnHCl and urea induced denaturation of PfGLP1 and the fragment Thr^{10} to Ile^{127} of PfGLP1

2 μM PfGLP1 or the fragment Thr^{10} to Ile^{127} were dissolved in 20 mM phosphate buffer pH 8.0 (containing 150 mM sodium sulphate, 1 mM DTT, 10% glycerol and 0.1 mM EDTA) in the absence and presence of increasing concentration of GdnHCl/urea and incubated for optimized time at 4 °C, before the measurements were made. The stock concentrations of GdnHCl and urea were made as described earlier (Tripathi, 2013).

Fluorescence Spectroscopy

Fluorescence spectra were recorded with a Perkin-Elmer LS50B fluorescence spectrometer in a 10 mm path-length quartz cell. The excitation wavelength for tryptophan was 285 nm and the emission was recorded from 300-400 nm. The excitation and emission slits were kept as 6 nm and 4 nm respectively. Each spectrum was corrected by subtraction of the corresponding blank sample without protein and the final spectrum was the average result of three independent scans.

CD Measurements

CD measurements were made with a Jasco J800 spectropolarimeter calibrated with ammonium

(+)-10-camphorsulphonate and attached to a Peltier thermostat. The results are expressed as θ_{obs} or CD. CD spectra were measured at an enzyme concentration of 2 μM at 25 °C in a 1 mm cuvette. For temperature-induced unfolding 10 mm path length cuvette was used. The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of denaturant under similar conditions. The final spectrum was the average result of three independent scans.

Thermal denaturation

Thermal denaturation experiments were performed by increasing the temperature from 30 to 100 °C and taking the CD readings at 222 nm in a 10 mm cuvette. Unfortunately, the protein was found to precipitate at the end of the experiment at high temperature. Due to this irreversible nature of the thermal transitions, further thermodynamic analysis of the data could not be performed. All data were corrected for the baseline contribution of the buffer at all conditions.

Results and Discussion

Preparation of PfGLP1 and the proteolysed fragment Thr^{10} to Ile^{127}

The expression of the recombinant PfGLP1 was good and the expressed protein was present predominantly (> 90%) in the soluble fraction. The protein present in the soluble fraction was purified by the method described earlier (Rahlfs *et al.*, 2001; Tripathi *et al.*, 2008). The yield of the purified recombinant reduced PfGLP1 was in the range of 3 to 5 mg/L. The native PfGLP1 was subjected to proteolysis with trypsin as explained before. The ESI-MS and SDS-PAGE of the purified native and proteolysed fragment Thr^{10} to Ile^{127} showed that the preparations were homogenous and more than 95% pure.

GdnHCl- and urea- induced denaturation suggests comparable stability of the full length and the fragment Thr^{10} to Ile^{127} of PfGLP1

The unfolding characteristic of the full length and fragment Thr^{10} to Ile^{127} of PfGLP1 was studied by monitoring the GdnHCl and urea induced changes in the secondary structure and tryptophan fluorescence of the proteins.

Time dependent changes in the structural parameters of full length and the fragment Thr^{10} to Ile^{127} of PfGLP1 at increasing GdnHCl concentrations (0.5, 1 and 4 M) were monitored to standardize the incubation time required to achieve equilibrium under these conditions. Under all the conditions studied, the changes occurred within a maximum of 3 h with no further alterations in the values obtained up to 12 h (Data not shown). These observations suggested that a minimum time of about 3 h is sufficient for achieving equilibrium under any of the denaturing conditions studied. Furthermore, under the experimental conditions the unfolding/ refolding curves were fully reversible. To study the GdnHCl- induced changes in the secondary structure of the full length and the fragment Thr^{10} to Ile^{127} of PfGLP1, far-UV CD studies were carried out. Figure 1 summarizes the effect of increasing concentration of GdnHCl on the CD ellipticity at 222 nm and tryptophan fluorescence of full length and the fragment Thr^{10} to Ile^{127} of PfGLP1. A sigmoidal loss of CD signal was observed between 2 to 5 M GdnHCl and at ~ 5 M GdnHCl almost complete unfolding of the protein was observed. Also on increasing the GdnHCl concentration from 2 to 6 M, a sigmoidal shift in emission wavelength maxima from 334 nm to 350 nm was observed (Figure 1), indicating the GdnHCl induced complete unfolding of the protein at about 6 M GdnHCl. From the results of loss of the CD signal at 222 nm and changes in tryptophan fluorescence emission maxima with increasing concentration of GdnHCl as reported above, a C_m of 3.3 M GdnHCl was found to be associated with unfolding of both the full length and the fragment Thr^{10} to Ile^{127} of PfGLP1. These observations demonstrate that GdnHCl induces cooperative unfolding of both the full length and N-terminal deleted fragment Thr^{10} to Ile^{127} PfGLP1 with similar efficiency.

The stability of the full length and the fragment Thr^{10} to Ile^{127} of PfGLP1 was also studied by measuring the CD ellipticity at 222 nm and tryptophan fluorescence as a function of urea concentration. Time dependent changes in the structural parameters of full length and N-

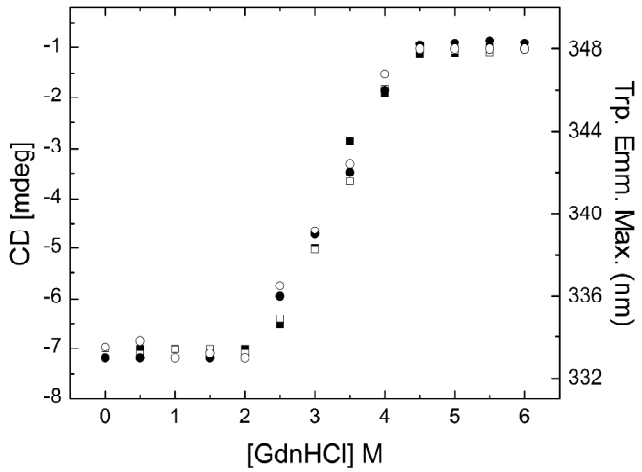

Figure 1: **GdnHCl-induced unfolding of native and N-terminal deleted fragment Thr[10] to Ile[127] of PfGLP1.** Square and circle solid boxes represent the CD signal at 222 nm and tryptophan fluorescence of native PfGLP1 while square and circle hollow boxes represents the CD signal at 222 nm and tryptophan fluorescence of N-terminal deleted fragment Thr[10] to Ile[127] of PfGLP1 respectively.

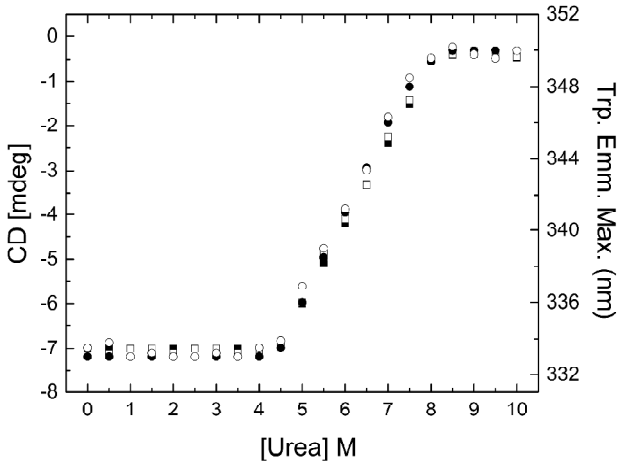

Figure 2: **Urea-induced unfolding of native and N-terminal deleted fragment Thr[10] to Ile[127] of PfGLP1.** Square and circle solid boxes represent the CD signal at 222 nm and tryptophan fluorescence of native PfGLP1 while square and circle hollow boxes represents the CD signal at 222 nm and tryptophan fluorescence of N-terminal deleted fragment Thr[10] to Ile[127] of PfGLP1 respectively.

terminal deleted PfGLP1 at increasing urea concentrations (2, 4 and 8 M) were monitored to standardize the incubation time required to achieve equilibrium under these conditions. Under all the conditions studied, the changes occurred within a maximum of 5 h with no further alterations in the values obtained up to 12 h (Data not shown). These observations suggested that a minimum time of about 5 h is sufficient for achieving equilibrium under any of the denaturing conditions studied. Under the experimental conditions the unfolding/refolding curves were fully reversible. Figure 2 summarizes the loss of CD signal at 222 nm and changes in tryptophan fluorescence emission at increasing concentration of urea for full length and fragment Thr[10] to Ile[127] of PfGLP1. An exponential loss of CD signal was observed between urea concentrations of 4 to 9 M urea. From tryptophan fluorescence also an exponential shift of fluorescence emission maxima from 334 to 350 nm was observed between urea concentrations of 4 to 9 M. These observations suggest that a complete denaturation of both the full-length and the fragment Thr[10] to Ile[127] of PfGLP1 is observed at 9 M urea with a C_m of approximately 6.5 M urea.

The GdnHCl- and urea-induced denaturation curves were used to determine the free energy of stabilization in the absence of denaturants (ΔG_D^{H2O}) by linear extrapolation of the ΔG_D values to zero denaturant concentration. The ΔG_D^{H2O} values of native and N-terminal deleted fragment Thr[10] to Ile[127] of PfGLP1 is shown in table 1.

Table 1
ΔG_D^{H2O} values of native and N-terminal deleted fragment Thr[10] to Ile[127] of PfGLP1. The values are given in kcal mol[-1]. Results are means ± S.D. of three independent measurements

	GdnHCl	*Urea*
Full length PfGLP1	3.20 ± 0.12	3.14 ± 0.14
Fragment Thr[10] to Ile[127] of PfGLP1	3.24 ± 0.15	3.18 ± 0.15

Thermal denaturation of full length and fragment Thr[10] to Ile[127] of PfGLP1

In order to study the effect of the N-terminal portion of PfGLP1 on the thermal stability of the protein, comparative thermal denaturation studies were carried out with the full length and the N-terminal truncated PfGLP1. The thermal denaturation was studied by monitoring the loss of the CD signal at 222 nm at increasing temperature and the results are summarized in Figure 3. For both the full-length and the truncated PfGLP1, a similar thermal denaturation curve having a Tm of about 72 °C was observed under the conditions of the study. However, both the proteins were found to precipitate at the end

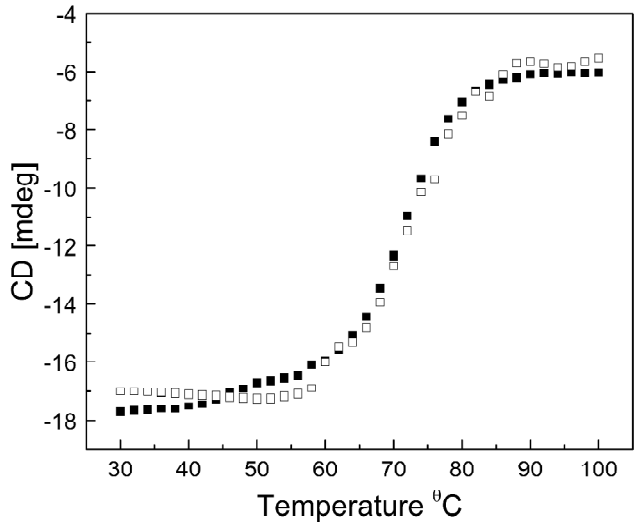

Figure 3: **Temperature-induced unfolding of native and N-terminal deleted fragment Thr[10] to Ile[127] of PfGLP1.** Solid boxes represents the native PfGLP1 while hollow boxes represent the N-terminal deleted fragment Thr[10] to Ile[127] of PfGLP1.

of the experiment at high temperature. Due to this precipitation, exact Tm associated with the thermal denaturation cannot be found out as with variable scan speed the Tm was found to shift. However, under similar conditions a similar thermal denaturation was observed for both the full length and the N-terminal truncated protein, suggesting that the N-terminal portion has no role in maintaining the thermal stability of PfGLP1.

Conclusions

The results presented in this paper demonstrate that in the native conformation of PfGLP1, the N-terminal portion is stabilized as a relatively flexible, open structure due to which it is easily accessible to protease for proteolysis. The unfolding studies showed that the PfGLP1 is a highly cooperative molecule as simultaneous unfolding of both the full length and N-terminal deleted fragment is observed during both the GdnHCl- and urea- denaturation studies. Comparative stability studies with full-length and N-terminal truncated PfGLP1 demonstrate that the N-terminal portion of PfGLP1 has no role in providing stability to the PfGLP1. Similar results from the stability of C-phycoerythrin and its N-terminal variant has been recently observed (Anwer et al., 2015). These results collectively suggest that the flexible N-terminal region containing few residues does not provide additional stability of certain enzymes and can be an evolutionary rudimentary region of the protein.

Abbreviations

Grx, Glutaredoxin; Trx, Thioredoxin, GLP1, Glutaredoxin like protein 1; PfGLP1, *Plasmodium falciparum* glutaredoxin like protein 1; GdnHCl, Guanidine Hydrochloride.

Acknowledgement

The study was supported by a research grant from Department of Biotechnology, Government of India to TT (Grant No. BT/28/NE/TBP/2010; 28.02.2011). The author dedicates this manuscript to his guide and mentor Late Dr. Vinod Bhakuni for showing him the light of scientific research. Author also thanks Prof Katja Becker and Dr. Stefan Rahlfs, Justus Leibeg University, Germany for providing the clone of PfGLP1 and for constructive suggestions.

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