

Research Article

ALLOSTERIC INHIBITION OF TRIOSE-PHOSPHATE ISOMERASE BY S-GLUTATHIONYLATION

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Abstract: S-glutathionylation regulates several cellular processes by modulating protein function. Many enzymes of the glycolytic pathway have been shown to be targets of S-glutathionylation, wherein S-glutathionylation results in inhibition of the pathway. In this study the effects of S-glutathionylation of triose-phosphate isomerase are reported. The kinetics and sites of S-glutathionylation of triose-phosphate isomerase were identified using high resolution mass spectrometric analysis and their consequences on enzyme activity were examined by *in vitro* biochemical assays. Combined analysis of data from biochemical assays and mass spectrometry have provided interesting insights into a possible novel mechanism of regulation of this enzyme by S-glutathionylation of cysteine-217 present in helix G of triose-phosphate isomerase.

Key words: Protein S-Glutathionylation; oxidative stress; metabolic regulation; triose-phosphate isomerase; allosteric inhibition.

Introduction

S-Glutathionylation refers to the specific post-translational modification of protein cysteine residues by the reversible covalent addition of glutathione. Glutathione, a tripeptide present at millimolar concentrations (1 – 10mM) inside the cell, is the most abundant and important non-enzymatic antioxidant defense in many organisms and helps to maintain the reduced environment of the cytosol. Extent of protein S-glutathionylation generally rises during oxidative / nitrosative stress but might also rise in unstressed cells under normal physiological conditions. Oxidative stress has been implicated in the pathogenesis of numerous diseases (Halliwell and Gutteridge, 1999). S-Glutathionylation has been shown to regulate a

variety of cellular processes by modulating protein function and to prevent irreversible oxidation of protein thiols (Klatt and Lamas 2000; Cooper *et al.*, 2011; Xiong *et al.*, 2011; Mieyal and Chock 2012). Proteomics based studies have identified many target enzymes of glutathionylation belonging to various cellular processes. The growing list of targets of S-glutathionylation include glycolytic enzymes, protein kinases, transcription factors, Ras proteins, heat shock proteins, ion channels and pumps, mitochondrial proteins and cytoskeletal proteins (Ghezzi *et al.*, 2005). Enzymes involved in various pathways of energy metabolism constitute the major target group of glutathionylation.

Reversible glutathionylation of proteins can rapidly shift the activity of a key metabolic enzyme and thereby result in dramatic, reversible changes in cellular metabolism. The glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase, aldolase, phosphoglycerate kinase, pyruvate kinase and

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Triosephosphate isomerase are confirmed targets of glutathionylation (Cotgreave *et al.*, 2002; Fratelli *et al.*, 2002; Dalle-Donne *et al.*, 2009; Michelet *et al.*, 2006; Ito *et al.*, 2003). Studies on glyceraldehyde-3-phosphate dehydrogenase, which has a role in apoptosis, has shown that glutathionylation of the highly reactive, active site cysteine leads to inhibition of the enzyme activity (Cotgreave *et al.*, 2002; Mohr *et al.*, 1999).

Triose-phosphate isomerase (TIM), a ubiquitous glycolytic enzyme that catalyzes the interconversion of glyceraldehydes-3-phosphate and dihydroxyacetone phosphate has been shown to be a target of S-glutathionylation (Ito *et al.*, 2003). The crystal structures of the enzyme from several different species are available and its kinetics is well established (Albery and Knowles 1976; Knowles 1991). The dynamics of the enzyme both during the resting state and during active catalysis has been thoroughly characterized (Rosovsky *et al.*, 2001; Cui and Karplus, 2002; Kurkcuoglu *et al.*, 2006; Waley, 1973). Only the dimeric form of the enzyme exhibits high catalytic rates, albeit each monomer has its own catalytic residues (Zabori *et al.*, 1980; Zomosa *et al.*, 2003; Fratelli *et al.*, 2003).

Though inhibition of TIM by S-glutathionylation has been reported in some species (Ito *et al.*, 2003; Marino *et al.*, 2010), the specific sites of S-glutathionylation and the relationship between site specific glutathionylation and activity have not been established. The two major determinants of the susceptibility of thiols to redox regulation are the accessibility of the thiol within the three-dimensional structure of the protein and the reactivity of the cysteine, which is influenced by the surrounding amino acids. Cysteine reactivity towards various sulfhydryl reagents is regulated by a number of factors which include – exposure to the solvent, dissociation of the thiol to the thiolate anion, reactivity of the SH reagent, charge compatibility between the reagent and the cysteine environment, stability of the bonds and the nature of the leaving group of the sulfhydryl reagent (Kiley and Storz, 2004). There are now several examples which show that a single cysteine modification can regulate protein function at various levels like enzyme activity and

protein-protein interactions. There are now emerging reports of proteins that contain cysteines susceptible to modification that can act as cellular protectors or regulators (Nakamura *et al.*, 1997; Reddie and Carroll, 2008., 2008; Rhee *et al.*, 2005; D'Autreaux and Toledano, 2007; Brandes *et al.*, 2009; Kumsta and Jakob, 2009; Foster *et al.*, 2009; Leitner *et al.*, 2009; Gharza-Ramos *et al.* 1996).

For homologous TIMs which possess a dimeric interface cysteine at position 13/14/15 in loop1 it has been shown that chemical modification of this cysteine by thiol reagents induces drastic changes in the quaternary (subunit dissociation) and tertiary structure which leads to abolition of catalytic activity. Examples of such homologous TIMs are found in species such as *Trypanosoma brucie*, *Trypanosoma cruzi*, *Leishmania mexicana* and *Entamoeba histolytica* (Borchert *et al.*, 1995). Several other studies have shown that mutations at the subunit interface of the protein destabilize the dimer leading to complete inactivation or drastic reduction in catalytic activity (Mainfroid *et al.*, 1996; Lambeir *et al.*, 2000; Gokhale *et al.*, 1999; Gopal *et al.*, 1999; Sampson and Knowles., 1992; Lodi *et al.*, 1994; Casal *et al.*, 1987).

For homologous TIMs which possess Cys-217 at the N-terminal of helix G, but lack the dimeric interface Cys-13, inactivation with thiol modifying reagents is achieved by local structural perturbation without any involvement of dimer dissociation, which suggests a possible communication between the region of the derivatized cysteine (Cys-217) and the catalytic site. Examples of such homologous TIMs include mammalian TIMs like rabbit TIM and human TIM. TIMs from certain parasite, plant and plant fungi species have been found to possess cysteines at both Cys13 and Cys217 (See Figure 1). Examples of such homologous TIMs are found in species like *Arabidopsis thaliana*, *Zea mays*, *Secale cereale*, *Plasmodium falciparum* etc. PfTIM contains four cysteine residues at positions 13, 126, 196 and 217 in each sub unit. Cys13 is present on loop1 at the interface and lies very close to the active site residues - Lys12, His95 and Glu165. Cys126 is located on β -strand 4, whereas Cys196 and Cys217 are found on helix F and helix G,

respectively of the TIM barrel (Velankar *et al.*, 1997) (Table 1). In a study on the differential reactivity of the four cysteine residues of PfTIM, at positions 13, 126, 196 and 217, towards thiol alkylating agents (IAA/IAM) was established using ESI-MS and was found to follow the order Cys196 > Cys13 >> Cys217/Cys126 (Maithal *et al.*, 2002).

The objective of the present study has been to identify the specific cysteine residues whose modification by S-glutathionylation lead to loss of enzyme activity. The studies have been carried out on recombinantly produced samples of PfTIM. The sites of glutathionylation have been identified by high resolution mass spectrometry and the effects

of glutathionylation on the activity of the enzyme have been corroborated through biochemical enzymatic assay. The relationship between sequence conservation across species and sites of glutathionylation has been explored and the mechanistic basis of inhibition has been proposed.

Materials and Methods

Iodoacetic acid (IAA), Iodoacetamide (IAM), 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB), oxidized glutathione, reduced glutathione, bovine trypsin, α -glycerophosphate dehydrogenase, glyceraldehyde-3-phosphate solution, NADH, Tris-HCl, triethanolamine, trifluoroacetic acid were purchased from SIGMA.

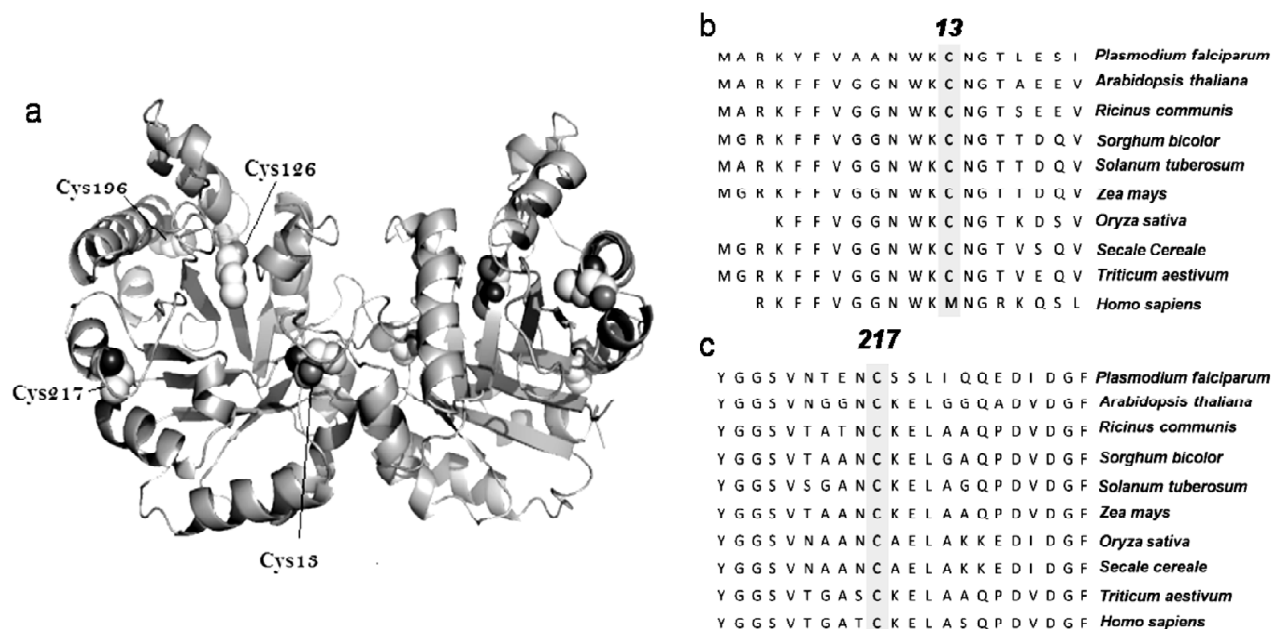


Figure 1: Cysteine residues in PfTIM.

a. Cartoon representation of dimeric PfTIM (PDB ID - 1YDV) showing the position of the cysteine residues. Cysteine residues (shown in sphere representation) of only monomer A have been labeled.

b. and c. Select portions of the sequence alignment of homologous TIMs which have high degree of sequence identity with PfTIM (with the exception of human TIM).

Table 1
Relative surface accessibility of cysteine residues in PfTIM

Residues	All-atoms	Side chain	Main chain	Non-polar	All-polar
Cys13	0.1	0.15	0.0	0.15	0.0
Cys126	0.0	0.0	0.0	0.0	0.0
Cys196	28.45	15.1	62.85	18.95	53.95
Cys217	3.6	5.0	0.00	4.95	0.0

Protein expression and purification

PfTIM was overexpressed in Terrific Broth media using a pTrc99A vector construct in the *E. coli* strain AA200, which has a null mutation in the host TIM gene. The induced cells were harvested and resuspended in 20ml of 100mM Tris-HCl (pH 8.0), 1mM EDTA and lysed using a French press. The protein was purified from the crude lysate by ammonium sulfate precipitation followed by Q-sepharose chromatography. The pure fractions were concentrated and the protein was further purified by gel filtration chromatography.

Mass spectrometry

LC-ESI

All LC-ESI-MS spectra were recorded on a HCT-Ultra ETDII ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). A linear gradient of increasing percentage of acetonitrile was used as the mobile phase at a flow rate of 0.4ml/min. For both protein samples and trypsin digested samples Agilent RP C18 reverse phase column was used for separation. The column was pre-equilibrated with 0.05% trifluoroacetic acid in water. The elution was performed over 60 min with a linear gradient of 5-90% acetonitrile in 0.05% trifluoroacetic acid. Data were acquired across a mass range of 125-4000 m/z using ion trap. Data processing was done using the deconvolution module of the Bruker Daltonics software to detect multiple charge states and obtain derived masses of the protein fragments. Tandem MS/MS experiments were carried out in positive ion mode by high energy CID using Helium as the collision gas. The fragmentations were carried out inside the ion trap through the collision of helium gas with the ions of interest, which were excited kinetically by increased resonance amplitude of the dipolar field, with a value typically set between 1 and 3V.

Preparation of glutathionylated proteins

Glutathionylation was carried out by incubating 20 μ M solution of protein at 37°C for varying time periods from 5 minutes to 60 minutes with 5mM oxidized glutathione (GSSG). Excess unreacted GSSG was removed by dialysis using microdialysis cassettes at 4°C. The

glutathionylated products were identified using LC-ESI-MS methods.

Trypsin digestion

20 μ M solution of PfTIM in Tris buffer pH 8.0 was subjected to trypsin digestion at 37°C for 24hr using 0.2 μ M solution of bovine trypsin. The same procedure was followed for glutathionylated PfTIM samples.

Enzyme assay

Enzyme assay for triosephosphate isomerase was carried out using a protocol described by Plaut and Knowles (Plaut and Knowles., 1972) in a JASCO UV double-beam spectrophotometer at 25°C. The cuvette contained 100mM triethanolamine buffer, pH 7.6, 1mM EDTA, 0.24 mM NADH, α -glycerophosphate dehydrogenase (50 μ g.mL⁻¹) and 1mM glyceraldehydes-3-phosphate. Enzyme activity was determined by monitoring the decrease in A₃₄₀.

Surface accessibility calculations

The program NACCESS (Lee *et al.*, 1971) version 2.1.2 was used to calculate the relative surface accessibility using a probe radius of 1.4Å water molecule.

Far UV-circular dichroism – Far UV-CD measurements were carried out on a Jasco J-715 spectropolarimeter. Ellipticity changes were monitored across a wavelength from 190 to 250nm using a path length of 1mm and the spectra were averaged over four scans at a scan speed of 5nm/min.

Size exclusion chromatography – Analytical gel filtration profiles were done on a Superdex™ 10/300 GL gel filtration column fitted to a Pharmacia (Uppsala) FPLC. The column was previously calibrated with standard proteins. The protein was eluted at a flow rate of 0.3 ml/min with 100mM Tris-HCl, pH 8.0.

Time course fluorescence measurements – Time course fluorescence measurements were recorded on a JASCO FP - 6300 spectrofluorometer at 25°C for a time period of 2hours after addition of 5mM oxidized glutathione. The excitation wavelength used was

295nm instead of 280nm to reduce contributions from tyrosine residues and the emission intensities were monitored at 331nm. The excitation and emission bandpasses were kept at 5nm.

Results

Mass spectrometric analysis

The kinetics of glutathionylation of cysteine residues of PFTIM were monitored using ESI-MS. Figure 2a shows the electrospray mass spectra of PFTIM taken at different time points after initiating labeling with GSSG. Each addition of glutathione will lead to a mass increase of 305 Da. The electrospray ionization mass spectra of PFTIM labeled with oxidized glutathione for just 10 minutes shows a singly labeled species (28,136 Da), corresponding to the addition of one glutathione moiety as the predominant species. A considerable amount of unlabeled species (27,831 Da) and some doubly labeled species were also detected. After 20 minutes of labeling the electrospray ionization mass spectra of labeled PFTIM showed a predominant doubly labeled species (28,441 Da) and a considerable amount of singly labeled species (28,136 Da). It was observed that at the end of 30 minutes a heterogeneous mixture of singly, doubly and triply labeled species were detected with triply labeled species (28,746 Da) being the predominant one. Figure 2b shows the time course of labeling of cysteine residues in PFTIM by oxidized glutathione.

To determine the sites of glutathionylation (Table 2), ESI-MS of peptide fragments generated by trypsin digestion of protein samples labeled with oxidized glutathione for varying time periods (0, 5, 10, 15, 20, 25 and 30 min) were recorded. The reverse phase HPLC profile revealed that the glutathionylated tryptic peptide fragments corresponding to labeling at Cys196, Cys13 and Cys217 eluted at 26.4min (480.8 m/z, doubly charged), 48.4 min (634.3 m/z, doubly charged) and 34.3 min (1231.0 m/z, triply charged) respectively. The identity of these tryptic peptide fragments were confirmed by subjecting the mass selected ion to MS/MS tandem experiment involving high energy CID. The resulting MS/MS spectrum of the peptide

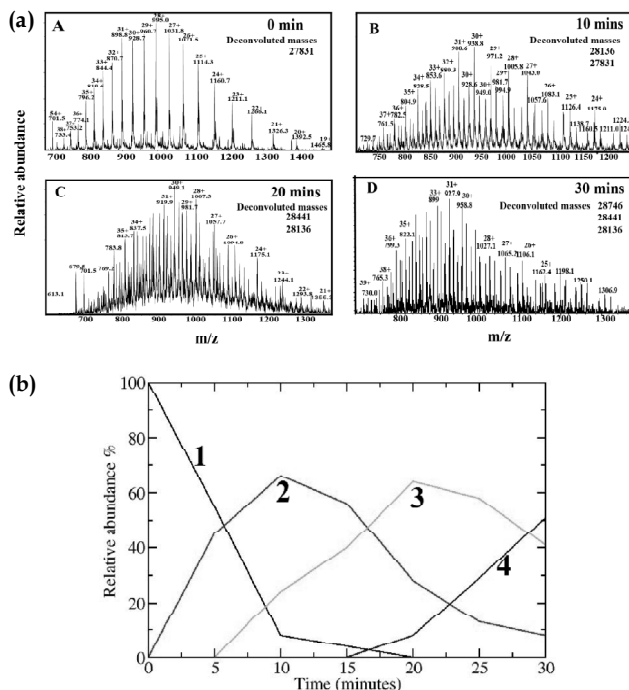


Figure 2: Kinetics of glutathionylation of PFTIM.

- Mass spectral analysis of the glutathionylation reaction** - The ESI-MS of differentially labeled PFTIM at different time points after addition of 5mM GSSG are shown. The deconvoluted masses of differentially labeled species which have relative abundance greater 50% are indicated in the figure.
- Abundance of differentially labeled species.** The relative abundance of differentially glutathionylated species of PFTIM after the initiation of the labeling reaction is shown. Curve 1 represents Unlabeled PFTIM, Curve 2 represents singly labeled PFTIM, Curve 3 represents doubly labeled PFTIM and Curve 4 represents triply labeled PFTIM. The proportions of the differentially labeled species were estimated directly from the observed intensities in the deconvoluted mass spectrum assuming that all of the species ionize with equal efficiency.

fragments corresponding to labeling at Cys196 (Asp194 to Lys199), Cys13 (Cys13 to Lys21) and Cys217 (Ile206 to Lys237) are shown in Figure 3, Figure 4 and Figure 5 respectively. The tryptic fragment Ala123 to Arg134 was detected without any modification (1337.5 m/z) at Cys126 even for samples labeled with GSSG for one hour (data not shown). From this timed study it was possible to establish the rate of glutathionylation to follow this order Cys196 > Cys13 > Cys217 >>> Cys126.

Rapid glutathionylation of Cys196 comes as no surprise when its surface accessibility is taken into account (Table 1). However Cys13 having almost zero surface accessibility shows high rate of glutathionylation. The high rate of glutathionylation of Cys13 was further confirmed

Table 2
Cysteine containing tryptic fragments analyzed by mass spectrometry.

Residues	Modification	Mass Theoretical	Sequence
13 - 21	Glutathionylation	1269.6	C(X)NGTLESIK
194-199	Glutathionylation	957.2	DTC(X)GEEK
206-237	Glutathionylation	3691.2	ILYGGSVNTENC(X)SSLIQQEDIDGFLVGNASLK

In the table glutathionylated cysteine is shown as C(X)

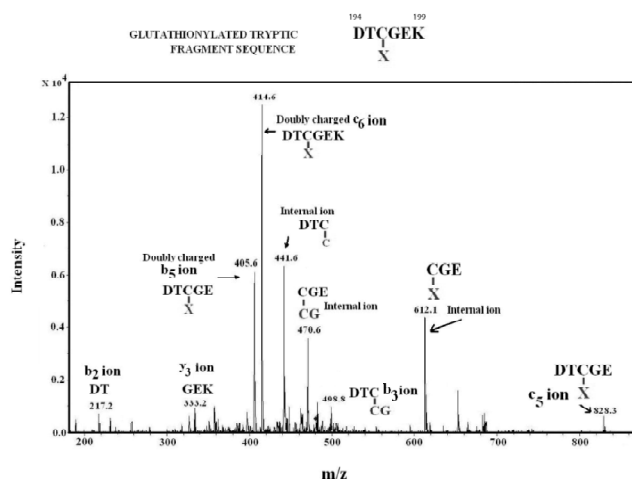


Figure 3: MS/MS analysis of glutathionylated tryptic peptide fragment Asp194 to Lys199.

Tandem mass spectra (MS/MS) for the doubly charged glutathionylated tryptic ion of DTC(X)GEEK (m/z 480.8). In the figure X represents covalently attached (disulfide bond) tripeptide glutathione.

by intrinsic tryptophan fluorescence time course experiment. Trp11 situated at the dimer interface and close to Cys13 contributes the maximum to the fluorescence intensity of PftIM (Figure 6a). Glutathionylation of Cys13 produces marked alterations in the intrinsic tryptophan fluorescence of wild type PftIM. When C13E mutant of PftIM was used glutathionylation had no effect on the intrinsic tryptophan fluorescence. Glutathione labeling of Cys13 was tracked using a time course fluorescence measurement as shown in Figure 6b. The intrinsic fluorescence of Trp11 decreases rapidly at a rate constant of 0.034 min^{-1} for nearly 30 minutes after which it plateaus. This may indicate that the Cys13 labeling leads to subunit dissociation which explains the Trp11 fluorescence quenching.

Size exclusion chromatography

The subunit dissociation was confirmed by size exclusion chromatography. After labeling for 20

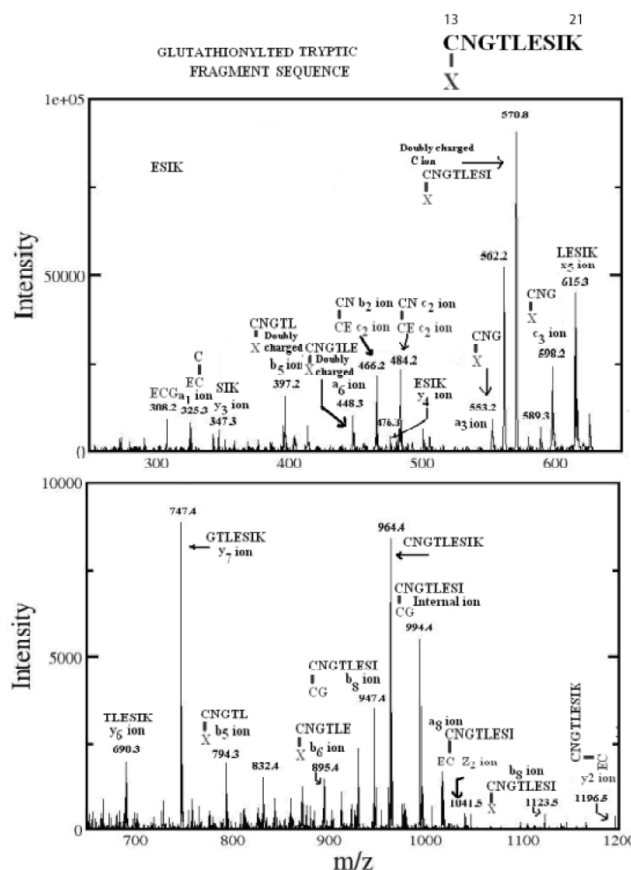


Figure 4: MS/MS analysis of glutathionylated tryptic peptide fragment Cys13 to Lys21.

Tandem mass spectra (MS/MS) for the doubly charged glutathionylated tryptic ion of C(X)NGTLESIK (m/z 634.3). In the figure X represents covalently attached (disulfide bond) tripeptide Glutathione.

minutes, by which time labeling of Cys13 reaches its maximum (Figure 2b), the differentially labeled protein was passed through a calibrated size exclusion gel filtration column to see the effect of labeling on the quaternary structure of the protein. Comparing the chromatograms for unlabeled and labeled protein it is evident that the appearance of a new peak at a higher elution volume corresponds to the monomeric species (Figure 7a and Figure 7b). The appearance of a

sharp gel filtration peak at a higher elution volume in the case of glutathione labeled enzyme suggests that the monomeric species generated due to subunit dissociation may be substantially folded. This is substantiated by comparing the CD signatures for the eluates corresponding to monomeric and dimeric species as shown in the Figure 7c. The glutathione labeling kinetics of PFTIM reported in this study closely corresponds with the IAM labeling reported from a previous study (Maithal *et al.*, 2002). However here the labeled monomer remains substantially folded and soluble but in the case of IAM labeling the monomer probably unfolds, aggregates and precipitates.

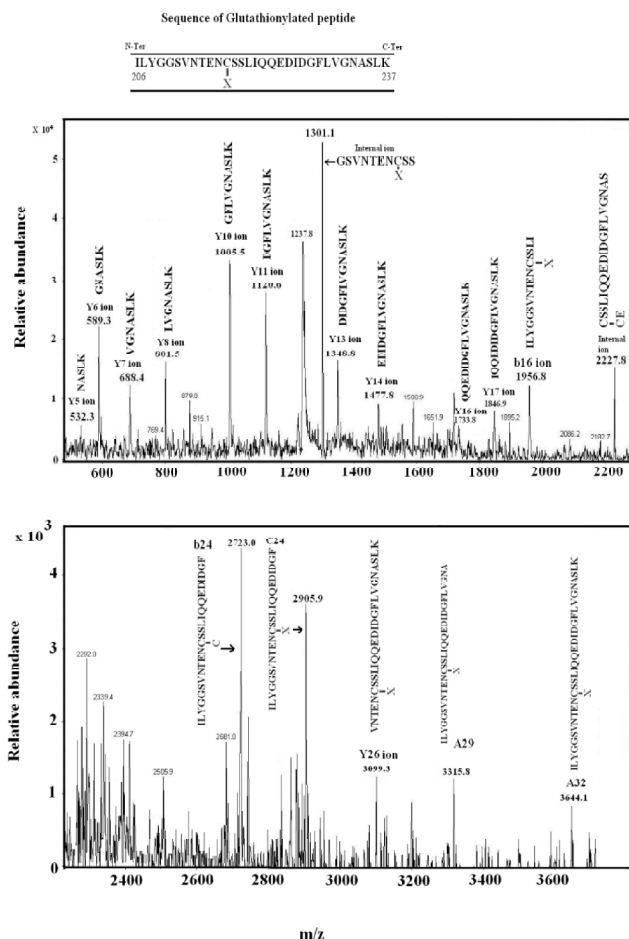


Figure 5: MS/MS analysis of glutathionylated tryptic peptide fragment Ile206 to Lys237.

Tandem mass spectra (MS/MS) for the triply charged glutathionylated tryptic ion of ILYGGSVNTENC (X)SSLIQQEDIDGFLVGNASLK (m/z 1231.0). In the figure X represents covalently attached (disulfide bond) tripeptide Glutathione.

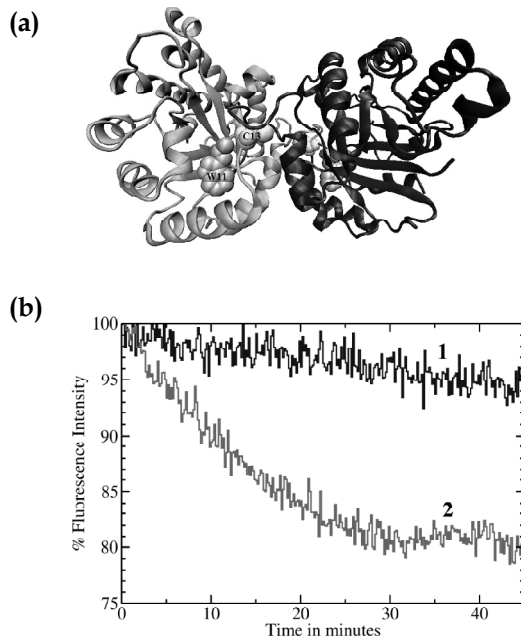


Figure 6: Time course fluorescence measurements of Glutathionylation of PFTIM.

- Cartoon representation of the dimeric enzyme PFTIM showing Trp11 and Cys13 in sphere representation across the dimer interface.
- Time Course Fluorescence measurements
 - 5µM protein solution in 20mM Tris-HCl, pH 8.0.
 - After addition of 5mM oxidized glutathione to 5iM protein solution in 20mM Tris-HCl, pH 8.0. Excitation wavelength used was 295nm and the fluorescence emission was monitored at 331 nm.

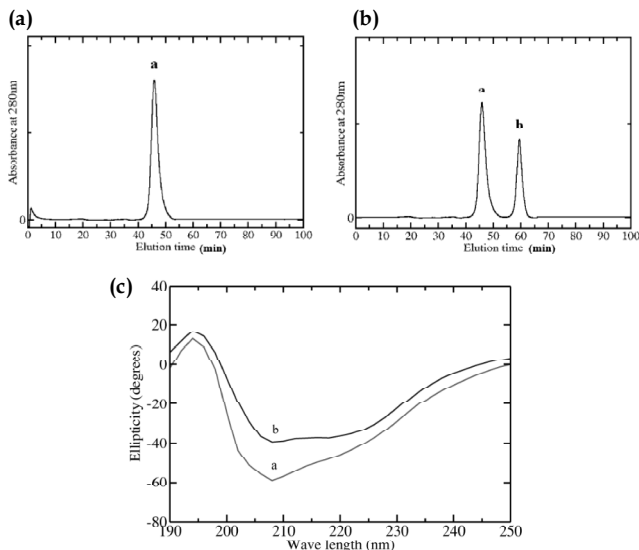


Figure 7: Size exclusion chromatography of glutathionylated PFTIM.

- Shown here is the size exclusion chromatogram of unmodified PFTIM.
- Shown here is the size exclusion chromatogram of PFTIM incubated with 5mM GSSG for 20 minutes.
- CD spectra of eluant species marked a and b from the above chromatogram.

Activity Assays

To find the susceptibility of PFTIM to inhibition by glutathionylation *in vitro* activity assays were done after incubating the protein at physiological temperature for varying time periods of incubation (10 to 30min). The effect of labeling on the enzymatic activity of PFTIM is shown in Figure 8a. The pseudo first order kinetics rate constant for loss of activity by glutathionylation of wild type PFTIM was found to be 0.022 min^{-1} . The time point corresponding to the maximum Cys196 labeling (10 min) was associated with less than 15% loss of activity. The time point corresponding to maximal Cys13 labeling (20min) was associated with over 60% loss of activity and the time point corresponding to maximal Cys217 labeling (30 min) was associated with complete loss of activity. This means that glutathionylation

of Cys196 has very little effect on enzyme kinetics/activity. The pseudo first order kinetics rate constant for loss of activity of C13E PFTIM due to glutathionylation was found to be 0.016 min^{-1} . IAM treatment of C13E mutant of PFTIM had no effect on its activity while it inhibited wild type PFTIM (Figure 8b)

Since redox signaling/regulation involves S-glutathionylation which is reversible, activity assays were done to check the effect of deglutathionylation on the enzyme activity. It was found that the glutathionylated wild type PFTIM could not be reactivated by deglutathionylation using reductants like DTT. However the C13E mutant could be completely restored to its initial level of activity upon treatment with DTT (Figure 8c). The time for half maximal reactivation with DTT was $\sim 20 \text{ min}$.

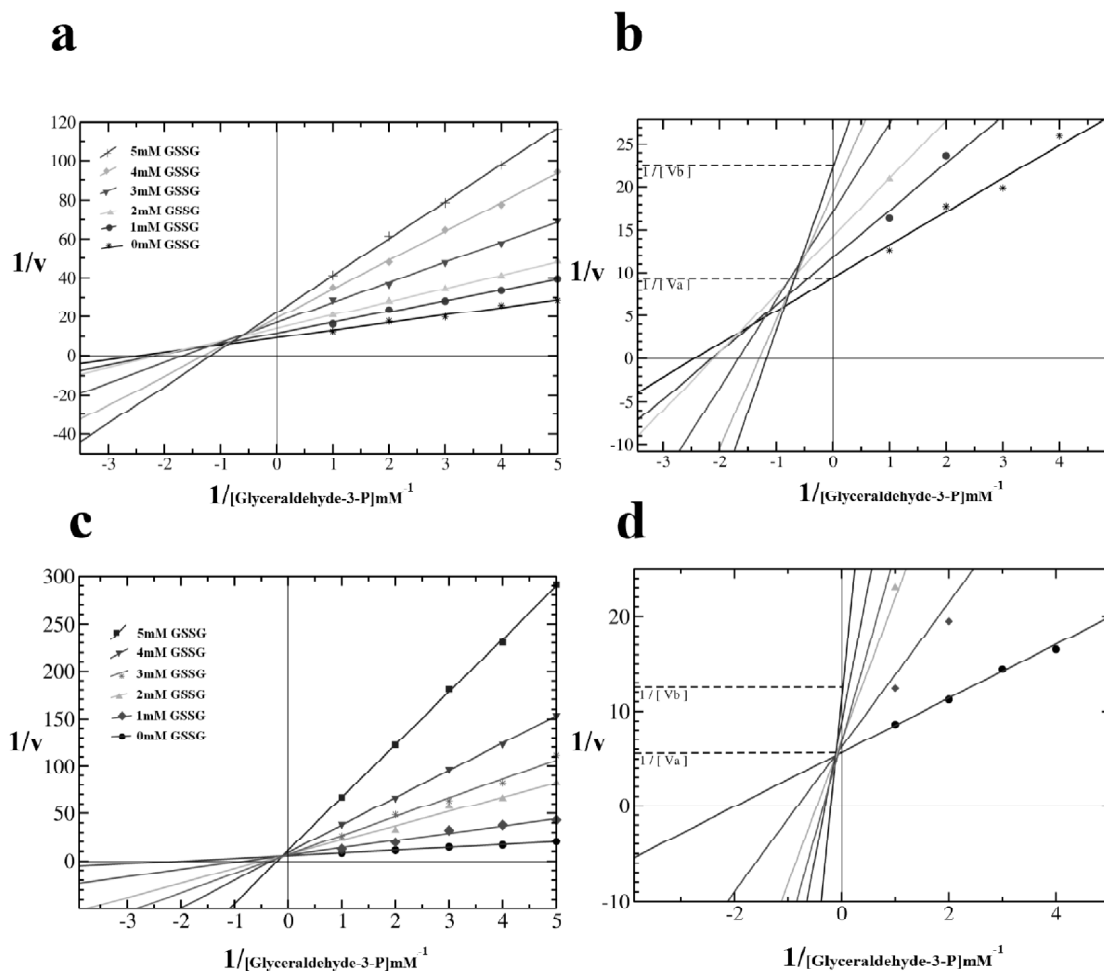


Figure 8: Double-reciprocal plots of GSSG inhibition

Double-reciprocal plots of GSSG inhibition of wild type PFTIM (a and b) and C13E PFTIM (c and d) at different GSSG concentrations (0 - 5mM). The figures on the right hand side are close up views of figures on the left hand side.

Discussion

Triose-phosphate isomerases across species show a very high structural similarity and are known to be catalytically active only in their homodimeric form. The TIM homodimer is very stable having nanomolar binding affinity and buries $\sim 1600 \text{ \AA}^2$ - 1800 \AA^2 of surface area per monomer. The dimer interface is stabilised by a number of polar and non polar interactions involving loop 1 residues from one subunit to the loop 3 residues from the other subunit. In PfTIM, Cys13 in loop 1 forms a number of interactions with loop 3 residues involving the backbone of Phe69, Asn71, Gly72, Ser73, Tyr74 and Glu77 of the other subunit. Cys13 is also involved in side chain-side chain interactions with Ser67, Glu77 and Ser79.

Cys13 which has only limited solvent accessibility in the native dimeric structure undergoes rapid modification with glutathione. The high rate of labeling of this residue may be due to contributions from its pKa, loop dynamics and possibly from electrostatic steering of negatively charged glutathione towards the positively charged surface surrounding the active site. Trp11 situated at the dimer interface provides an opportunity to selectively study Cys13 labeling kinetics. It contributes the maximum to the fluorescence intensity of PfTIM. The intrinsic tryptophan fluorescence of Trp11 decreases rapidly at a rate constant of 0.034 min^{-1} for nearly 30 minutes after which it plateaus. The quenching of intrinsic tryptophan fluorescence should be due to solvent exposure of Trp11 as a result of Cys13 labeling. In rabbit TIM it has been shown that derivatization of Cys217 affects the kinetics of the enzyme, but it did not modify its spectrum of intrinsic fluorescence at an excitation wavelength of 280nm, furthermore inhibition of enzyme activity was fully removed by DTT (Garza-Ramos *et al.*, 1996).

The observation of two well resolved and separated distinct peaks corresponding to the dimeric and monomeric species in the gel chromatography profile of a 20 minute labeled protein suggests that the reassociation rate constant k_{on} is negligible compared to k_{off} (i.e. no equilibrium exists). This suggests that as the interface cysteine gets labeled the subunit-subunit

interactions are perturbed to an extent that the dimer dissociates. Glutathionylation may hinder monomer association in PfTIM by sterically blocking the formation of certain interface contacts or by causing local structural rearrangements in these monomers that impede formation of interface contacts. A similar loss in enzymatic activity on derivatization of the interface cysteine with the sulfhydryl reagent methyl methanethiosulfonate has been reported from other parasitic TIMs from *T. brucei*, *T. cruzi*, and *L. mexicana* (Perez-Montfort *et al.*, 1999). In a previous study involving the subunit interface mutant Y74G distinct peaks corresponding to both dimeric and monomeric species have been observed (Maithal *et al.*, 2002).

The glutathione labeling kinetics of PfTIM reported here closely corresponds with the IAM labeling of PfTIM reported from a previous study (Maithal *et al.*, 2002). A notable difference is that in the case of glutathione labeling the labeled monomer retains significant amount of fold and remains soluble whereas in the case of IAM labeling the monomer unfolds, aggregates and precipitates. Reactivation of Cys13 labeled wild type PfTIM was not possible because spontaneous reassociation of monomers does not happen after deglutathionylation under dilute *in vitro* conditions and may also be indicative of some level of unfolding. However inside the cell there exists a possibility of reassociation of monomers upon deglutathionylation of Cys13 to form once again a functional dimer under suitable conditions. This means that glutathionylation / deglutathionylation of Cys13 could serve as a potential dimer-monomer switch of redox regulation of parasitic TIMs which possess this dimer interface cysteine.

Correlating the rate of loss of activity as deduced by activity assays with the glutathionylation kinetics deduced from ESI-MS experiments indicates that glutathionylation of Cys13 and Cys217 play a significant role in enzyme inhibition.

Comparing the rate of loss of activity with time for the wild type PfTIM and C13E PfTIM indicates that rate of Cys217 glutathionylation does not lag too far from that of Cys13 glutathionylation.

Activity assays with C13E PFTIM suggests that in the absence of Cys13, inhibition does happen at the same GSSG concentration but with a little slower rate of loss of activity (0.016 min^{-1}).

The activity assays along with mass spectrometric study of differentially labeled TIM have unequivocally established the crucial role of Cysteine-13 and Cysteine-217 glutathionylation in enzyme inactivation.

The fact that the ESI-MS shows that by the end of 20 minutes the trilabeled species appears which corresponds to Cys217 labeling, as determined by the tryptic digest studies, indicates that the Cys217 labeling may also play a role in the inactivation of the enzyme even in the presence of Cys13.

Activity assays with the C13E mutant of PFTIM showed that the enzyme loses around 20% of its activity by 15 minutes and 85% of its activity by 30 minutes. Thus it is clear that glutathionylation of Cys217 leads to appreciable loss of activity. This leads to the conclusion that the glutathione labeling of Cys217 is the principal reason for the inactivation of the enzyme in the absence of dimeric interface cysteine-13.

The complete reversibility of activity by glutathionylation / deglutathionylation of Cys217 reinforces the potential significance of allosteric modulation of activity through this residue. Cys217 situated at the N-terminal of Helix G has been shown to frequently undergo helix transitioning between α to 3_{10} forms in the presence of substrate. In Glycerol-3-phosphate bound yeast TIM, residues at the N-termini and C-termini of helix G – Asn213, Phe220 and Lys221 were found to have ^{15}N chemical exchange rate constants (R_{ex}) significantly above zero indicative of slow conformational exchange process on the μs time scales (Massi *et al.*, 2006). Recently it has been shown that residues in helix-G are involved in protein-protein interactions with redox proteins like thioredoxin (Hameed and Sarma., 2012). The dynamic nature of redox interactomes requires fast and transient molecular switches and therefore the underlying recognition motifs are usually short segments that are structurally malleable. Recognition through linear motifs results in interactions with μM affinities

that underlie transient, reversible complexes, adapted for effective regulation. They also serve as consensus sites of post-translational modification and recognition elements in transient complexes (Fuxreiter *et al.*, 2007).

The mechanism of inhibition by glutathionylation of Cys-217 appears purely kinetic and therefore can be readily reversed by deglutathionylation. However there was no loss of activity when C13E mutant PFTIM was treated with IAM. This means that carboxy-methylation of Cys-217 does not render the enzyme inactive while glutathionylation of Cys-217 renders the enzyme inactive. In previous studies it has been shown that although Cys217 does not form part of the catalytic site, its transformation to a phenyl disulfide exerts important effects on the kinetics of the enzyme (Garza-Ramos *et al.* 1996). This suggests that modification of Cys-217 with bulky thiol reagents renders the enzyme inactive. It has been shown in *Giardia lamblia* TIM that derivatization of Cys222 (equivalent to Cys217 of PFTIM) significantly decreased the affinity for the substrate (Flores *et al.*, 2011) and led to very high K_m . Reversibility by DTT of S-phenyl-p-toluenethiosulfonate action in other TIMs that have a Cys217 has been observed.

Thus it is clear that S-glutathionylation can cause reversible allosteric inhibition of TIMs that lack Cys13, but otherwise have Cys217; those that lack Cys13 and Cys217 will be hardly affected by S-glutathionylation.

The fact that glutathionylation of Cys217 leads to loss of activity is interesting as in mammalian TIM Cys217 is conserved and further human TIM has methionine instead of Cys13. This may be very interesting as regulation of human TIM by reversible glutathionylation of Cys217 may have implications in cancer. There are many parallels that can be drawn between malarial parasites and tumour cells (Ginsbury., 2010; Zhang., 1987). Both of them are heavily dependent upon glycolysis for their energy requirements and avoid mitochondrial respiration as a source of energy (Warburg effect). This may help to prevent oxidative stress to some extent as mitochondrial respiratory chain is a powerful source of reactive oxygen species. Both *P. falciparum* and tumour cells are particularly susceptible to oxidative

challenge and thiol based redox systems involving glutathione system and the thioredoxin system play crucial roles in antioxidant defense. Drugs which target thiol based redox enzymes and glutathione metabolism would cripple the antioxidant defense system of the parasite/tumour cell and would lead to widespread inhibition of many target enzymes involved in various processes of metabolism.

Further it has been shown in T-lymphocytes that human TIM, which possess Cys-217 but lacks dimeric interface cysteine, is a target of S-glutathionylation during oxidative stress conditions (Fratelli *et al.*, 2002). Thus glutathionylation/deglutathionylation of Cys-217 could effectively regulate the activity of TIM and provides a novel mechanism whereby ROS/RNS can modulate S-glutathionylation and regulate this key glycolytic enzyme.

Conclusion

In this study it has been demonstrated that S-glutathionylation of Cys13 and / or Cys217 results in inhibition of TIM by using mass spectrometric analysis and biochemical assays. This study leads to the conclusion that homologous TIMs that lack Cys-13 but have Cys-217 can potentially be regulated by ROS/RNS via S-glutathionylation through reversible allosteric modulation of enzyme kinetics. This constitutes a novel mechanism of redox regulation of Triosephosphate isomerase.

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Abbreviations

TIM, Triosephosphate isomerase; PftIM, *Plasmodium falciparum* Triosephosphate isomerase; LC-ESI-MS, Liquid Chromatography-Electrospray ionisation-Mass spectrometry; MALDI, matrix-assisted laser desorption / ionization; EDTA, Ethylene diamine tetraacetic acid; RP-HPLC, Reverse phase

- High performance Liquid chromatography; DTT, Dithiothreitol; TCA, Tricarboxylic acid; DHAP, Dihydroxy acetone phosphate; G3P, Glyceraldehyde-3-phosphate; ATP, Adenosine triphosphate; GSH, reduced glutathione; GSSG, oxidized glutathione.

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