Discussion

As the world grows smaller through increasing economic integration and travel, health is becoming a global issue, particularly in the realm of parasitic diseases. Parasitic organisms are a major cause of disease worldwide, but because of their concentration in poorer parts of the world, very little attention has been paid by the pharmaceutical industry and major scientific funding agencies. Four (Malaria, Leishmaniasis, Chagas disease, African trypanosomiasis) out of the ten diseases targeted as research priorities by the World Health Organization's Special Program for Research and Training in Tropical Diseases (http://www.who.int/tdr/), are caused by protozoan parasites. The development of drugs, vaccines and diagnostics, can be pursued from an evolutionary perspective, taking advantage of comparative genomics to identify and exploit characteristics that distinguish pathogen and host. Contrary to popular opinion, there is no shortage of effective antibiotics, but those that kill the patient are not particularly useful. From this perspective, eukaryotic pathogens pose a more difficult challenge than prokaryotes, as they share many metabolic pathways with humans.

A series of papers in 2005 issues of Science report have shown, the complete genome sequences of three important protozoan parasites- of the phylum Kinetoplastida—*Leishmania major* (Ivens et al., 2005), *Trypanosoma brucei* (Berriman et al., 2005), and *T. cruzi* (El-Sayed et al., 2005). Comparative study of the genome of these parasites promises to elucidate the biology of many devastating tropical diseases, and helped in identifying parasite-specific targets for drug development. Recent advancements in the identification and understanding of genome sequence of Leishmania parasite have provided new impetus to find novel drug targets. *L. major* genome sequencing showed that its 32.8-megabase haploid genome had 36 chromosomes, which predict 911 RNA genes, 39 pseudogenes, and 8272 protein-coding genes, of which 36% can be ascribed as a putative function. The mean length of genes was approximately 2045bp and total G+C

content was 57.3 % (Ivens et al., 2005). The availability of the entire genetic content of one Leishmania species provides the foundation for the identification and in-depth functional analysis of virulence factors, critical enzymes in key metabolic pathways, and potential vaccine candidates. Beside this it also provide crucial information for the development of new therapies for the leishmaniasis. Metabolic pathways have traditionally provided attractive targets such as Dihydrofolate Reductase in Plasmodium, Trypanothione Reductase in Leishmania for drug development and are therefore of particular interest. (Chaudhary and Roos, 2005).

Extensive studies of metabolism in African trypanosomes leave no doubt that the bloodstream stage uses glycolysis as its only source of ATP. Inhibition of glycolysis therefore leads to rapid death of these parasites The same will possibly be true for *T. cruzi* and Leishmania spp. which, despite quantitative differences in the contribution of glycolysis to the overall free-energy supply compared to *T.brucei*, also utilize glucose as a major energy substrate (Clayton and Michels, 1996; Tielens and Van Hellemond , 1998; Cazzulo,1992) Moreover, the long evolutionary distance between trypanosomatids (Fernandes et al., 1993) their mammalian hosts, and the unusual organization of the glycolytic pathway in the parasites (Opperdoes and Borst, 1977) have endowed the trypanosomatid enzymes of glycolytic pathway with distinct properties (Lakhdar-Ghazal et al., 2002). As better understanding of biochemical pathaways that would be helpful in the development of new antileishmanial, we exploited glycolytic pathway of *L. donovani*. Triosephopshate isomerase is the important enzyme of glycolysis which interconverts glyceraldehyde 3- phopshate to dihydroxyacetone phopshate. TIM function is essential for the net production of ATP in glycolysis; therefore, this enzyme represents a promising target for drug design against glycolytic pathogens.

The trypanosome triosephosphate isomerase was the first glycolytic enzyme of parasite to be crystallized and its three-dimensional structure was solved (Wierenga et al., 1991; Wierenga et al., 1984). In the bloodstream form of *T. brucei*, the TIM enzyme is mainly found in the glycosome and it represents less than 0.04% of the total trypanosome protein (Misset et al., 1986). Sandra Helfert and Christine Clayton (University of

Heidelberg) have tried the double knock-out of the triosephosphate isomerase (TIM) gene in *T. brucei*. One TIM gene could easily be removed, the second gene could not, and therefore, had to be placed under the control of a regulatable promoter. A reduction of the level of expression led to a reduction in the rate of growth, and its complete suppression led to growth arrest. One possible explanation for this is that TIM inhibition or removal, would force the bloodstream form of *T. brucei* to switch to anaerobic glycolysis with a net production of only one molecule of ATP per molecule of glucose consumed rather than two, as under aerobic condition and the limited amount of ATP synthesized under these conditions is not sufficient to sustain cell division and growth so TIM is also a vital enzyme and may serve as an attractive therapeutic target (Helfert et al., 2001).

TIM is the only glycolytic enzyme found in glycosomes for which no peroxisome-import signal has been reported so far. Steaphane de Walque (ICP, Brussels) has studied its import into glycosomes and showed that a 21 amino-acid-long internal peptide has the ability to route a reporter protein to glycosomes. Replacement of this peptide by a corresponding peptide of the yeast TIM completely abolished import. He proposed that this peptide, which is exposed at the surface of the native protein, mediates the interaction of TIM with another protein that does carry a peroxisomal targeting signal (Opperdoes and Michels, 2001). Recently the internal sequence was identified in *T. brucei* which targets the TIM to glycosomes (Galland et al., 2010). The polypeptide is 22 amino acid long fragment ranging from 140-161.

Till date there are no reports available in literature of cloning, overexpression and characterization of TIM from *Leishmania donovani* a causative agent of visceral leishmaniasis. Keeping this in view the present study was undertaken. *L. donovani* gene encoding triosephosphate isomerase was successfully cloned, expressed and characterized. Substantial differences with the mammalian host were found suggesting TIM may be an attractive target for chemotherapy. The results presented herein are a first step towards a better understanding of the biochemical, biophysical and structural properties of TIM in *Leishmania donovani*. Our findings further support the feasibility of specifically targeting Leishmania TIM through rational drug design.

5.1. Cloning and Sequencing of LdTIM

Selection of parasite enzymes as drug targets is usually based on its functional importance and conservation across different species. The TIM gene from *L.mexicana*, *T. brucei*, *T.cruzi*, *Giardia lamblia*, *Plasmodium falciparum, Mycobacterium tuberculosis and* Human etc has been cloned and characterized (Kohl et al., 1994; Borchert et al., 1993; Ostoa-Saloma et al., 1997; Enriquez-Flores et al., 2008; Ranie et al., 1993; Mathur et al., 2006; Mande et al., 1994). The cloned TIM gene from *T. brucei*, *L. mexicana* and *Giardia lamblia* has been expressed and purified through conventional method (Borchert et al., 1993; Kohl et al., 1994; Lopez-Velazquez et al., 2004). It had opened new avenue to characterize this enzyme at crystal structure and other biochemical and biophysical level. However no such reports are available on heterologous expression of *L. donovani* TIM in *E. coli.* Thus there is an urgent need for obtaining the large quantity of the leishmanial TIM to elucidate its biochemical, biophysical and structural functional relationship, as well as large screening of antileishmanial compounds on HTS for the development of new drugs.

A 753 bp amplicon of TIM was amplified from the *L. donovani* (Dd8) genomic DNA and cloned in PCR vector (pGEMT easy). The DNA sequence for coding region was found rich in G + C residues (61.9 %) as reported for other leishmanial gene. Amino acid sequence analysis showed that LdTIM has all the conserved four amino acid involved in the catalytic mechanism i.e lysine 13, asparagine 12, glutamic acid 168 and histidine 96. The asparagine 12 and lysine 14 residues are involved in substrate binding while glutamic acid 168 and histidine 96 are involved in catalysis

The Protein sequence alignment of LdTIM reveals the presence of a consensus pattern A-Y-**E**-P-V-W-A-I-G-T-G among all the TIM genes mentioned in results. The highly conserved motif is same as the reported TIM consensus signature [AVG]-[YLV]-**E**-P-[LIVMEPKST]-[WYEAS]-[SAL]-[IV]-[GN]-[TEKDVS]-[GKNAD] (**E** is the active site residue) (PROSITE access number: PS00171). The percentage amino acid homology between TIMs of different organisms is summarized in Table 5.1.

	LdTIM	LmaTIM	LiTIM	LmTIM	TbTIM	TcTIM	PfTIM	HTIM
LdTIM	XXX	93	92	88	68	66	43	48
LmaTIM	93	XXX	98	92	68	68	43	49
LiTIM	92	98	XXX	90	69	68	41	48
LmTIM	88	92	90	XXX	69	68	44	49
TbTIM	68	68	69	69	XXX	73	41	51
TcTIM	66	68	69	68	73	XXX	41	50
PfTIM	43	43	43	44	41	41	XXX	42
HTIM	48	49	48	49	51	50	42	XXX

Table 5.1. Percentage amino acid homology of LdTIM and TIMs from other organisms

The deduced amino acid sequence of the LdTIM protein shows ~90% homology to TIMs from other Leishmania species; 68% and 66 % homology with TbTIM and TcTIM and 48 % with HTIM. All other Leishmania species also shows ~ 66-68 % homology with TbTIM and TcTIM and ~ 48-49 % homology with HTIM which shows that the LdTIM gene, isolated via its homology with the LmTIM gene, is a typical TIM gene. It specifies a protein of the same size as other TIMs.

The region in the periphery of the interface of TcTIM has the capacity to bind two hexane molecules (Gao et al., 1999) as well as a benzothiazole derivative that causes inactivation of the enzyme (Tellez-Valencia et al., 2004). This portion of the interface was examined in TIMs from different species. The data showed that this portion of the interface is strikingly similar in the enzymes from the trypanosomatids and differs markedly from that of the non-trypanosomatids. This portion of LdTIM was compared to the equivalent regions of other TIMs as shown in Table 5.2.

TcTIM	LdTIM	TbTIM	LmTIM	HTIM	PfTIM
Ile 66	Ile	Ile	Ile	Tyr*	Ser*
Arg 71*	Lys	Lys	Lys	Val*	Phe*
Phe 75	Phe	Phe	Phe	Phe	Tyr*
Tyr 102	Tyr	Tyr	Tyr	Val*	Tyr
Tyr 103	Tyr	Tyr	Tyr	Phe*	Phe*
Gly 104	Gly	Gly	Gly	Gly	His*
Ile 109	Ile	Ile	Ile	Leu*	Asp*
Lys 113*	Thr	Lys*	Lys*	Lys*	Lys *

Table 5.2. Residues in the interface region of TcTIM (* represents the differences with LdTIM)

TcTIM has six residues while TbTIM and LmTIM have seven residues identical to those of LdTIM. In the other enzymes, the number of identical residues is lower: two in human and one in PfTIM when compared with

LdTIM. The portion of interface region in LdTIM differs from TcTIM at position 71 and 113. The comparison of this portion of LdTIM with HTIM shows that out of eight residues only two residues are found identical hence by targeting this portion of the interface; it would be feasible to find molecules that affect LdTIM but not TIM from Human.

In TbTIM glutamine 65 is completely buried in the dimer interface and participates in an intersubunit hydrogen-bond network (Wierenga et al., 1987). Substituting glutamine 65 in LmTIM for a glutamate yielded a protein with considerably greater thermostability than the wild-type. This glutamine residue is considered as weakening of intersubunit interactions in Leishmania species (Williams et al., 1999; Lambeir et al., 2000).

A highly specific inactivation in this enzyme was reported; the most studied non-conserved region of TIM from parasites is the interface formed by the two subunits. The chemical modification (derivatization) of Cys residues in the interface results in structural alterations and the irreversible abolition of TIM activity from *Trypanosoma cruzi* (TcTIM) (Maldonado et al., 1998), *T. brucei* (TbTIM), *Leishmania mexicana* (LmTIM) (G´omez-Puyou et al., 1995) , *Plasmodium falciparum* (PfTIM) (Maithal et al., 2002), and *Entamoeba histolytica* (EhTIM) (Rodrıguez-Romero et al., 2002) whereas the interface of TIM from human (HuTIM) contains Met instead of Cys and is found resistant to Cys-reactive compounds (Mande et al., 1994). LdTIM also has an interfacial Cys residue at position 15. The structural similarities and differences existing in the interfaces of oligomeric enzymes may be targeted to find agents that selectively inhibit the enzymes from different organisms (Enriquez-Flores et al., 2008).

The TIM based genotyping tool suggest that TIM gene is a good phylogenetic marker for analysis of the molecular evolutionary and taxonomic relationship (Baruch et al., 1996; Lu et al., 2002; Lu, et al., 2002).

5.2. Overexpression and Purification of LdTIM

Leishmania proteins are generally insoluble in nature and tend to form aggregate and appears as an inclusion bodies upon expression in prokaryotic hosts, e.g., adenylate kinase 2 (Villa et al., 2003) and methionine adenosyl transferase (MAT 2) of *L. donovani* (Perez-Pertejo et al., 2003), cysteine protease of *L. infantum* (Rafati et al., 2003), glucose regulating

protein 94 (GRP 94) of *L. infantum* (Larreta et al., 2000). To obtain functional protein from inclusion bodies is a tedious process and requires lot of laboratory work and time. Many researchers have tried several conditions to get active protein in the soluble form but the yield was too low to work upon. In practice, it is worthwhile to test several different vector/host combinations to obtain the best possible yield of protein in its functional form. We tried vectors having T7 promoters like pET-23(a) and pET-43.1(a) transformed in different *E. coli* strains like, BL21(DE3), Rosetta, BL21(DE3) pLysS etc. We observed that although the level of expression of recombinant LdTIM in pET-23(a)-TIM construct was very high but all the expressed recombinant protein aggregated and appeared in inclusion bodies.

To obtain LdTIM in soluble and active form it was further sub cloned in pET-43.1(a) vector having NusA tag. The strategy consisted of linking a solubility tag, NusA, to the target protein and expressing the resulting fusion protein at optimal favorable condition. Approximately 50 % recombinant LdTIM was present in soluble form when pET-43.1(a)-ldTIM construct transformed in BL21 (DE3) grown at 18° C, and induced with 0.1 mM IPTG. This soluble protein was purified through Ni-NTA column chromatography. The yield of purified protein was 5 mg/L culture. However, fusing the target LdTIM to a 55 kDa Nus-tag lacked functionality of the enzyme. Similar results were reported on cyclomaltodextrinase, that when it was expressed with Nus-tag it shows decrease in its specific activity (Turner et al., 2005). The possibility for loss of activity of LdTIM in pET- 43.1(a) vector is that, this vector has an extra 55kDa Nus-tag for solubility or May be the catalytic site of LdTIM buried inside during process of *in vivo* folding in *E. coli*.

We were able to obtain the soluble and active protein only in pET-28(a) vector. Decreasing the culture temperature up to 20°C and increasing the incubation time for 12 hours after induction with 1mM IPTG resulted in most efficient expression of LdTIM in soluble form (Fig. 4.2.9A, Lane- 5) which was further confirmed by western blot (Fig. 4.2.9B, Lane- 4). The purified recombinant TIM has a subunit mass of ~ 28 kDa as estimated by SDS-PAGE and western blot. It was in perfect accordance with the expected size from translation of nucleotide sequence. However it was 27.5 kDa in *L. mexicana* (Kohl et al., 1994), 27 kDa in *T. cruzi* (Ostoa-Saloma et

al., 1997) and *T. brucei* (Borchert et al., 1993), 28 kDa in *G. lamblia* (Lopez-Vela´zquez et al., 2004) and ~ 30 kDa in *M. tuberculosis* (Mathur et al., 2006). The purified LdTIM shows specific activity of 8.7 x 10^3 µm/min/mg while LmTIM shows a specific activity of 5.5 x 10^3 µm/min/mg (Kohl et al., 1994) and 5.7 x 10^3 µm/min/mg for *Mycobacteria tuberculosis* TIM (Mathur et al., 2006). Similar data were obtained from TbTIM (Borchert et al., 1993) and TcTIM (Ostoa-Saloma et al., 1997) that shows a specific activity of 5915 µm/min/mg and 7498 µm/min/mg respectively. Recombinant HTIM have a specific activity of 9800 µm/min/mg. Table 4.2.1 depicts the specific activity and fold purification at various steps during LdTIM purification. The two step purification lead to 8.7 fold purification. Total recovery was 4 mg/ 200mg wet weight of cells. The LdTIM was stable in Tris (Hydroxymethyl) aminomethane pH 7.6 buffer as no aggregation, degradation, or loss of the enzyme activity, were observed even when the purified LdTIM was stored at 4 °C for 2 months. The soluble functionally active enzyme was substrate specific and other kinetic parameters in accordance with the natural enzyme and the reported enzyme from other TIMs of parasite.

5.3. Oligomeric Properties of LdTIM

TIM is reported as a dimer in all known species except for the enzymes in *Thermotoga maritima* and *Pyrococcus woesei* where it is tetrameric (Maes et al., 1999; Walden et al., 2004). The SDS–PAGE of purified recombinant LdTIM showed a single band with an estimated size of 27.7 kDa (Fig.4.2.9A, Lane-6) based on the amino acid sequence (Fig.4.2.3 B) of the recombinant protein while on gel filtration (Fig.4.2.18A) the peak was observed at 54 kDa. Enzyme activity was observed in peaked fraction. Our data are in agreement with earlier observations, where only the dimeric form of the enzyme has been found to be active, even though each monomer has its own catalytic site (Zomosa-Signoret et al., 2003). Similar results were obtained with EhTIM (Landa et al., 1997), LmTIM (Kohl et al., 1994), Taenia solium TIM (TsTIM) (Jiménez et al., 2000), Giardia lamblia TIM (GlTIM) (Enriquez-Flores et al., 2008) and TcTIM (Ostoa-Saloma et al., 1997) that shows peak corresponding to dimer at 53.3 , 44.8, 48.5, 50 and 55 kDa respectively. The disruption of the dimeric form of the enzyme has thus been the focus for selective drug designing (Jackson and Phillips, 2002).

5.4. Enzyme Activity

Kinetic parameters for TIM catalysis of the elimination reaction of an equilibrium mixture of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP) to form methylglyoxal and phosphate ion are reported for the rabbit enzyme (Richard, 1991). Methylglyoxal is formed as a by-product of the TIM catalyzed reaction. Once formed, methylglyoxal irreversibly modifies amino groups in lipids, nucleic acids and proteins, forming advanced glycation end products. It is therefore toxic, mutagenic and an inhibitor of glycolytic enzymes. Enhancing methylglyoxal formation by understanding the biochemical properties of TIM will lead to harmful effects on trypanosomatids that might be exploited for therapeutic purposes. To develop new anti-parasitic drug against potential enzyme target, it is important to generate detailed information about its structure, biochemical and biophysical properties.

The pH stability profile has been determined by incubating the enzyme at 25°C in buffers of ionic strength 50 mM, having different pH values (4 - 11). The LdTIM was observed stable over a pH range of 7.2 to 9.0. Similar results were reported by Linda Kohl (Kohl et al., 1994) for the LmTIM and TbTIM (Borchert et al., 1993) and same was found stable over a wide pH range from 6.5-8.5.

The LdTIM enzyme showed maximum activity at 25°C, similar results were reported for other characterized TIMs (Kohl et al., 1994; Mathur et al., 2006; Borchert et al., 1993; Enriquez-Flores et al., 2008; Rodríguez-Romero et al., 2002). Thermostability of enzyme was estimated by measuring the activity, before and after 30 min of enzyme incubation at various temperatures in 50 mM Tris buffer (pH 7.6). The LdTIM remain stable for 30 minute up to a temperature of 37°C whereas reported data shows that LmTIM is more thermostable than LdTIM. The LmTIM was stable upto 30 minute at temperature 42°C (Kohl et al., 1994) while *Mycobacterium tuberculosis* TIM is stable up to 50°C (Mathur et al., 2006). Dependence of TIM activity on metal cofactors has not been reported from any source.

The conformational stability of LdTIM enzyme has been investigated in urea and guanidinium chloride (GdmCl) solutions using enzyme activity (Fig. 4.2.12). The activity of the protein is more sensitive

towards GdmCl than that of urea; this might be due to the ability of GdmCl to disturb the electrostatic interactions. The loss of LdTIM activity can be either due to unfolding of the enzyme, due to disruption of active site microenvironment in the presence of denaturant molecules or due to preferential binding of molecules on the surface of LdTIM. A biphasic curve corresponding to two independent transitions was observed in presence of both urea and GdmCl suggesting that first transition includes the unfolding of dimer into monomer whereas second transition reflects the unfolding of monomers. The thermodynamic parameters of chicken mucle TIM during its urea induced unfolding were as DeltaG degrees =3.54 kcal.mol($^{-1}$), and m(G) = 0.67 kcal.mol($^{-1}$)M($^{-1}$), which reflect the unfolding of dissociated folded monomer to fully unfolded monomer transition. The cooperative unfolding ratio, DeltaH (cal)/DeltaH(vH), was measured close to 2, indicating that the two subunits of chicken muscle TIM unfold independently. These results support the unfolding mechanism with a folded monomer formation before its tertiary structure and secondary structure unfolding (Shi et al., 2008). Our data is also in agreement with TbTIM that shows two intermediates in the unfolding of TbTIM induced by GdmCl, (Chanez-Cardenas et al., 2002).

The K_m for LdTIM at glyceraldehyde 3- phosphate concentration was 0.328 mM. The estimated K_m of LdTIM is similar to the K_m data reported for TIM of LmTIM (Kohl et al., 1994;) but higher than the K_m of TIM from its human counterpart (Gracy et al., 1975; Repiso et al., 2002; Mande et al, 1994). The kinetic constant of TIM from other sources is presented in Table 5.3.

Organisms	K_m (mM)	V_{max} (µm/min/mg)	Reference
LdTIM	0.328	10500	
LmTIM	0.30	5500	Kohl et al., 1994
TbTIM	0.9	7498	Borchert et al., 1993
TcTIM	0.25	5915	Ostoa-Saloma et al., 1997
EhTIM	1.05	-	Landa et al., 1997
TsTIM	0.37	2792.8	Jiménez et al., 2000
GlTIM	0.53	-	Lo´ pez-Vela´ zquez et al., 2004
HTIM	0.49	-	Mande et al., 1994

Table 5.3. Comparison of the K_m and V_{max} values of LdTIM with other TIMs

A higher K_m of LdTIM than HTIM suggests that the LdTIM enzyme probably has a more open active site pocket compared with that of human TIM. TIM enzymes show a high degree of conservation of the active site. However, outside the active site there is considerable sequence variation, which may be responsible for such structural differences leading to catalytic variations between enzymes from various sources. Thus, the active site of LdTIM differs from that of the human enzyme, and it would be interesting to study the extent of the variation and its implications for the design of effective inhibitors that target enzyme activity.

5.5. Inhibitory Studies

Species-specific inhibition of TIM enzyme has been reported for *T. brucei, T. cruzi, L.mexicana, Giardia lamblia* (Garza-ramos et al., 1998; Olivares-Illana et al., 2006; Enriquez-Flores et al., 2008). Sulphydryl reagents produce the irreversible inactivation of TIMs from parasites. We chose MMTS, a relatively small polar molecule that reacts with accessible cysteines forming a methyl disulfide, to study whether selective inhibition of TIMs having a cysteine in position 15 could be achieved. LdTIM has four cysteine residues at position 15, 40, 118 and 127.

The crystal structure of TbTIM showed that the cysteine residue at position 14 is important for the dimer stability (Wierenga et al., 1987). Cys14 is located at the beginning of the loop (loop 1) that joins the first β sheet and first α helix of triosephosphate isomerase; it forms part of the intersubunit contacts between the two monomers. In the crystal structure of TbTIM, Cys14 is at an H-bond distance (0.38 nm) from Ser71, Gly72, Glu77, Val78 and Ser79 of loop 3 of the other monomer. All five residues are conserved in TcTIM, LmTIM and LdTIM. The central role of Cys14 and that of other residues at position 14 in the stability of triosephosphate isomerase is well illustrated by several independent lines of research. The substitution of Cys14 by a leucine residue in TcTIM (Go´mez-Puyou et al., 1995) showed that derivatization of Cys14 by MMTS produced important structural alterations that lead to derivatization of the otherwise hidden cysteines. These structural changes also alter the environment of aromatic residues, and

abolish catalytic activity. Cys 39 and Cys 126 are inaccessible to MMTS (Garza-ramos et al., 1996).

In our studies the exposure of LdTIM to MMTS resulted in strong inhibition of its activity. The inhibition was observed in concentration and time dependent manner. LdTIM activity was 75 % inhibited at 250 µM MMTS concentration in 15 minutes suggesting LdTIM is more sensitive to MMTS than LmTIM and TbTIM. MMTS inhibited 80% of LmTIM and 90% of TbTIM activity at concentration of 1000µM and 400µM respectively. In TcTIM 100 % inactivation was achieved with in 15 minutes at 15 µM concentration (Garza-ramos et al., 1998). The different response to MMTS could be related to differences in the magnitude of the perturbations induced by modification of a given cysteine (Olivares-Illana et al., 2006). The reason for such a high inactivation of TcTIM is given by Georgina Garza-Ramos (Garza-ramos et al., 1998) that TcTIM has proline residue at position 23 near to cysteine residue, at this position the leishmanial and the TbTIM enzymes have a lysine residue and glutamic acid residue, respectively. One of the possible reason may be this proline residue of the TcTIM that affects the stability and the accessibility of MMTS to Cysteine residue (Garza-ramos et al., 1998). In accordance with this data LdTIM has leucine and isoleucine at position 29 and 33 respectively which is near to the Cys 15, at this position LmTIM and TbTIM has phenylalanine while TcTIM has leucine residue as similar to LdTIM. At position 33 the LmTIM and TcTIM have thereonine whereas TbTIM has serine. These residues are non conserved and may be responsible for difference in magnitute of MMTS action as compared to other trypanosomatids.

It was not surprising to find that derivatization of Cysteine of triosephosphate isomerase from LdTIM, TcTIM, TbTIM and LmTIM brought marked structural alterations. These differences very likely indicate that each of these enzymes has particular structural arrangements which provide hindrances of different magnitudes to the influx of derivatizing agents to Cys14. The results illustrates that markedly similar proteins with identical or similar cysteine contents exhibit dramatic differences to sulfhydryl reagents. The effect of MMTS on rabbit, Yeast and *E.coli* TIM was also reported. The MMTS reagent at 25 µM inhibited the activity of the rabbit enzyme

approximately 20 %; higher concentrations of MMTS or longer preincubation times did not increase the extent of inhibition. This could be due to a derivatization of one or more of the cysteines of rabbit TIM (Gomez-Puyou et al., 1995). *Saccharomyces cerevisiae* and *E. coli* TIM was completely insensitive to the MMTS reagent because they lack this cysteine residue (Garza-ramos et al., 1996). All the published reports showed the alteration of cysteine residue present at dimer interface leads structural perturbations and loss of catalytic activity.

5.6. Effect of MMTS on the Kinetics of LdTIM

The kinetic analysis showed that the K_m of LdTIM treated with MMTS was 340 µM whereas untreated LdTIM it is 328 µM indicating that MMTS does not affect the rate of catalysis. Similar data were obtained in GITIM (Enriquez-Flores et al., 2008) and trypanosomal TIM (Gomez-Puyou et al., 1995; Garza-ramos et al., 1998) where only slight difference in K_m value was observed in presence of MMTS.

5.7. Spectroscopic Characterization of the Recombinant LdTIM

The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Most of the emissions are due to excitation of tryptophan residues, with a few emissions due to tyrosine and phenylalanine. The buried tryptophan residues in folded protein show fluorescence emission maxima at around 330–335 nm. The unfolded protein shows λ_{max} at around 350nm with decrease in fluorescence (Lackowicz, 1983). The fluorescence spectra of LdTIM showed a peak at 338 nm indicating that tryptophan molecule(s) are not completely buried in the native protein but partially exposed to the solvent. This result is in agreement with mycobacterium TIM that gives peak at 340 nm in fluorescence spectra due to having partially exposed tryptophan molecules in native protein. Our studies on LdTIM show λ_{max} at 354.3 nm at 8 M urea concentration with decrease in fluorescence from 80 RFU to 20 RFU suggesting the exposure of tryptophan residues to the solvent or unfolding of LdTIM. Similar pattern was obtained in TcTIM (Vázquez-Contreras et al., 2004; Chanez-Cardenas et al., 2005).

5.8. Structural Changes in LdTIM Induced by MMTS

To achieve species-specific inhibition of homologous enzymes, we focused on non-conserved amino acids; if one of these residues or its integrity is essential for the function or stability of the enzyme, it should be possible to find agents that discriminate between homologous enzymes. We determined the alteration of non-conserved Cys l5 of LdTIM produced loss of catalytic activity. Specific inhibition of purified TIMs, that have this amino acid was achieved using the sulfhydryl reagent MMTS. Crystal structure resolved in TbTIM (Wierenga et al., 1987) showed Cys14 forms part of the intersubunit contacts between the two monomers. It is located ~ 4.3 A from the nearest molecular surface, and is adjacent to Lys l3, which is a conserved amino acid that lies at the catalytic site and serves to bind the substrate. The side chain of Cys l4 contacts the interdigitating loop of the other subunit of the TIM homodimer. It was shown that this loop is essential for dimer stability; modification of the loop gave rise to monomers (Borchert et al., 1994). LdTIM homodimer dissociated into monomers when treated with MMTS (Fig. 4.2.18). Our data is in agreement with EhTIM and GlTIM where dimer gives rise to monomers when they are treated with MMTS (Enriquez-Flores et al., 2008; Rodr´ıguez-Romero et al., 2002).

It was observed that when LdTIM treated with MMTS, increase in fluorescence was observed (Fig.4.2.19). Similar increase in ANS fluorescence was reported after incubation of TcTIM with MMTS or 5, 5-dithiobis-(2-nitrobenzoate) (DTNB), and for TbTIM and LmTIM incubated with DTNB (Gaza-ramos et al., 1998) indicating that structural alterations accompanied enzyme inhibition. Our data also is in agreement with EhTIM where ANS did not bind to native protein while increase in fluorescence was observed when EhTIM was treated with MMTS (Rodr´ıguez-Romero et al., 2002).

5.9. Refolding Studies of TIM

Expression of cloned genes in bacteria is widely used both in industry, for the production of pharmaceutical proteins, and in research, for the production of proteins for structural and/or biochemical studies. Bacteria produce large quantities of recombinant proteins in rapid, often inexpensive, fermentation processes; however, the product of interest is frequently

deposited in insoluble inactive aggregates or inclusion bodies. The general strategy used to recover active protein from inclusion bodies involves three steps: firstly, inclusion body isolation and washing; secondly, solubilization of the aggregated protein, which causes denaturation; and finally, refolding of the solubilized protein. While the efficiency of the first two steps can be relatively high, folding yields may be limited by the production of inactive misfolded species as well as aggregates. Formation of off-pathway species, such as incorrectly folded species and aggregates, are the cause of decreased renaturation yields. Because aggregation is an intermolecular phenomenon, it is highly protein concentration dependent. The most direct means of minimizing aggregation is by decreasing protein concentration. It has been suggested that optimum recovery yields can be expected if the protein concentration is in the range of 10–50 µg/ml (Rudolph and Lilie, 1996).

The manner in which a newly synthesized chain of amino acids transforms itself into a perfectly folded protein depends on both the intrinsic properties of the amino acid sequence and multiple contributing influences from the crowded cellular milieu (Dobson, 2003). Protein molecules, however, all have a finite tendency either to misfold or to fail to maintain their correctly folded states under some circumstances (Dobson, 2004). Thus correct folding and misfolding is a competing process during protein folding in intracellular environments. As shown in a study (Dedmon et al., 2003) an intrinsically disordered protein FlgM gains structure in living *Escherichia coli* cells and under physiologically relevant conditions *in vitro*, which suggests a reason for the observation that some proteins are only folded under physiologically relevant conditions and proves the biological relevance of studying proteins *in vivo* and at physiologically relevant solute concentrations. However, some proteins do not become structured *in vitro* at physiologically relevant solute concentrations, which may require another protein to provide a framework for structure formation. For example, certain transcription factors remain disordered even in solutions containing high concentrations of macromolecular crowding agents, yet they gain structure in the presence of other components of the complex (Flaugh and Lumb, 2001). It should be pointed out that *in vitro* simulations of macromolecular crowding *in vivo* do not necessarily provide results that are more meaningful in a biological context,

although they may be appropriate in certain specific experiments. Significant nonspecific interaction is an unavoidable consequence of crowding and confinement in most or all physiological fluid media (Minton, 2001).

Several TIM-barrel proteins are extensively studied in terms of folding and re-activation (Dubey et al., 2007; Dubey et al., 2005). Our data suggests that BSA induces the structural changes in denatured LdTIM, which resulted in refolding and regain of LdTIM enzyme activity by providing framework for structure formation. Similar data were obtained with TbTIM (Rodríguez-Almazán et al., 2007) indicating that TIM dimers have the capacity to interact with a wide variety of proteins.

This would be in consonance with reports that show that TIM interacts with actin, microtubules and membranes (Walsh et al., 1989; Knull and Walsh, 1992; Orosz et al., 2001; Orosz et al., 2000) and that TIM binding to the later structures is affected in human TIM deficiencies (Orosz et al., 2001). Likewise, it has been shown (Dhar-Chowdhury et al., 2005) that TIM, as well as other glycolytic enzymes, interacts with the K_{ATP} channel of the plasma membrane and thereby regulates its function. Thus, the collective data indicate that LdTIM is promiscuous that it interacts with several unrelated proteins in intracellular compartment to get its native form.

5.10. Molecular Modeling

Elucidation of the 3D structure of enzymes and correlating it to their function is one of the prime aims of modern biologists. The energy minimized and refined 3D structure of the LdTIM was built by using homology modeling based on the known crystal structure of TIM from *L. mexicana* (PDB code 1AMK) as a template. The quality of our LdTIM model has been checked using three different criteria. Our studies suggest that the backbone conformation (PROCHECK), the residue interaction (ProSA II) and, the z-score values are well within the limits established for the reliable structures. These tests suggest that we have obtained a good model for LdTIM to further characterize its binding site and explore its interactions with the substrate. The Lys 14 residue which was found outside the allowed region in Ramchandran plot suggests a unique constraint around the binding site. This lysine residue is conserved along the family and plays a important role in binding of substrate. Site directed mutagenesis studies on TbTIM revealed

that Lys 13 corresponding to Lys 14 of LdTIM is involved in catalytic activity (Schliebs et al., 1996).

A number of residues form the hydrophobic core of the 3D model structure of LdTIM. Most of these residues are either conserved or replaced by similar residues. There is a discrepancy for residue Ala 125, a hydrophobic residue in 1AMK that is replaced by polar residue Thr 126 in LdTIM.

Hence we conclude that triosephosphate isomerase of *L. donovani* (Dd8 strain) was successfully expressed in *E. coli* prokaryotic system. The recombinant enzyme is found biochemically active and could be exploited for structural functional studies, drug designing and as drug target.