This thesis describes biophysical, kinetic, structural, and sequence conservation analysis of the glycolytic enzyme triosephosphate isomerase (TIM). TIM catalyses the diffusion limited isomerization of D-glyceraldehyde-3-phosphate (D-GAP) and dihydroxyacetone phosphate (DHAP). The cloning, kinetic, biophysical and structural characterisation of Leptospira interrogans (Li) TIM; the mutagenic studies on the active site residue Phe96 and the dimer interface residue Tyr74 of the Plasmodium falciparum (Pf) cytoplasmic TIM; and the cloning, expression and preliminary characterization of the Plasmodium falciparum apicoplast TIM are reported.

Due to its unique biochemical properties and a highly stable structure, the enzyme triosephosphate isomerase has been at the centre of several kinetic, mechanistic and structural studies. TIM catalyses the isomerization reaction following a presumably simple acid-base mechanism. Despite seven decades of extensive work, some of the aspects of the enzyme have remained elusive. Chapter 1. provides a brief introduction of the enzyme “TIM/ TPI” with the aim to highlight the pertinent questions that were unanswered at the time of taking up this study. Taking TIM as the example, I will also describe the use and benefits of sequence conservation analysis when it is not straightforward to uncover the structure function relationship of residues.
Chapter 2. describes the cloning and expression of TIM from the zoonotic pathogen, *Leptospira interrogans*, and the relevance of TIM in this organism’s biochemistry. The chapter presents the crystal structure of the enzyme in its *apo* and *holo* (substrate bound) forms and draws attention to the structural determinants of stability, high reaction rate and reaction specificity achieved by TIM. The mechanistically relevant differences in the non-catalytic residues observed in *LiTIM* are discussed in the context of the widely accepted “classical mechanism” proposed by Knowles. Structural stability of the protein was probed by monitoring the change in the circular dichroism and fluorescence spectra after treatment of the protein with urea and guanidine hydrochloride. Also, a detailed study of temperature dependence of activity and stability of the three mesophilic, pathogenic TIMs from *Plasmodium falciparum*, *Leptospira interrogans* and *Trypanosoma brucei* was carried out. While, *PfTIM* was structurally more robust to thermal denaturation, it showed lower increment in activity with rise in temperature as compared to the other two enzymes. With the help of comparative analysis of the sequences and the structures of the three enzymes, I have tried to understand the basis of the observed differences and present a rationale for the same.

In Chapter 3, I have addressed the role of a highly conserved active site residue at position 96 using *PfTIM* as the model enzyme. In an overwhelming number of the sequences available for TIMs till the time of analysis, this residue was found to be Ser. *PfTIM* provided a unique opportunity to address this question since the plasmodial TIM is the only known eukaryotic TIM with a Phe replacement at this position. Residue 96 is a part of a highly conserved peptide stretch 94-GHSERR-99, with His95 and Glu97 being two of the catalytic residues. Also, residue 96 side chain makes contact with another catalytic residue Glu165. It is remarkable that all of the cytoplasmic TIM genes reported from the 12 plasmodial species have a Phe at this position. Given these facts, two questions arise— 1) why do only plasmodial TIMs have a Phe at position 96? And 2) how does a Phe residue in plasmodial sequences replace for the function performed by a small polar residue Ser in the rest of the TIMs? Biochemical and structural studies on the *PfTIM* mutants F96A, F96Y, F96S/S73A and F96S/L167V were carried out to address these questions and to probe the role of residue 96 in the catalytic cycle of TIM.
The importance of dimer integrity for function has long been studied in the enzyme triosephosphate isomerase. In the current study, *Plasmodium falciparum* TIM was the choice of model enzyme owing to the presence of a Cys (at position 13) at its dimer interface. Chapter 4. presents the successful engineering of a disulfide bridge at the interface of PfTIM and examines its effect on the activity and the structure of the enzyme. By use of site directed mutagenesis, the mutant Y74C was generated and the mutant remained a non-covalent dimer (albeit unstable compared to the wild type protein) in presence of a reducing agent while, upon oxidation, gave a covalently linked dimer with a symmetrical pair of disulfide bridges, Cys13 (A)-Cys74 (B), and Cys13 (B)-Cys74 (A). Covalent linking of the dimer substantially increased the thermal stability of the mutant, but it impaired the activity of the protein by decreasing the $k_{\text{cat}}/K_m$ by ~80 fold compared to that of the wild type protein. X-ray crystal structure of the oxidised form of the protein revealed that while, the overall architecture of the protein had remained the same, the dimer interface was reorganized. Introduction of a pair of covalent bonds at the interface constrains the conformation of the adjacent residues, resulting in their repositioning and loss of the native interface interactions. This further resulted in a change in the backbone and side chain conformation of Lys12, ionization state of His95 and movement of Glu97 side chain and loss of activity in the mutant.

Chapter 5. pursues the interesting observation of disulfide bond (C74-C13) cleavage in the absence of any reducing agents when the oxidized form of the mutant was denatured with chaotropes. The tempting possibility of a disulphide exchange reaction was ruled out by generating double and triple mutants, where the three other Cys residues present in the protein were systematically removed. The pH dependence of the reaction, peptide mass fingerprinting of the oxidised, reduced and the chaotrope denatured forms substantiated the hypothesis that the reaction was an outcome of attack of a His on the disulfide bond to generate dehydroalanine and cysteine persulfide. Further more, the experiments with His modifying reagents strengthened the proposition that His is involved in the disulfide cleavage reaction.

*Plasmodia* belong to the apicomplexan class of protozoan parasites which have a relict chloroplast like organelle, called the apicoplast. The plasmodial genome contains several proteins with an apicoplast targeting signal at their N-terminus, apicoplast-TIM
(ApicoTIM) being one of them. Chapter 6. presents the cloning of the *apicotim* gene using the m-RNA pool from the schizont stage of the parasite. In addition to the expected full length m-RNA, an unanticipated, slightly smaller form was also observed and was confirmed to be a splice variant after sequencing. Both the forms of the gene were sub-cloned for His-tagged expression. After expression and purification, the longer, full-length form of the protein showed extensive precipitation. Size exclusion chromatography confirmed the presence of higher order oligomers. Non-reducing denaturing PAGE of the protein and ESI/MS further helped to confirm the presence of disulphide bonds being the cause for the higher order oligomer formation. Preliminary biophysical and kinetic characterization of the protein was carried out and the enzyme was found to be around 8-9 fold less active in catalysing the isomerization in both directions *ie.*, from D-GAP to DHAP as well as from DHAP to D-GAP.

In Chapter 7. presents a comprehensive summary of the studies in this thesis and attempts to highlight how the results help furthering our understanding of the inter-relationship of structure, function and sequence conservation in the enzyme.