X-ray crystallographic studies of triosephosphate isomerase

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CHAPTER 10

SUMMARY

Triosephosphate isomerase (TIM) is an enzyme of the glycolytic pathway which catalyses the interconversion of dihydroxyacetonephosphate and D-glyceraldehyde-3-phosphate. The reaction catalysed by TIM has been highly characterised at the biochemical level, and therefore the enzyme is an ideal target for structural analysis, in order to achieve understanding of the structure function relationship. TIM is a dimer of identical subunits. Each subunit consists of a regular barrel like fold, formed from a (loop-β-loop-α)8 repeat, which is seen in many other proteins serving a diverse range of functions. Detailed study of TIM from a range of sources might help to shed light on the sequence-structure determinants of this fold, and therefore be useful in the design of proteins having novel structural or catalytic properties. The investigations described in this thesis concern X-ray crystallographic studies upon TIM from *Trypanosoma brucei* and *Escherichia coli*. TIM from the organism *Trypanosoma brucei* is of additional interest as a target for the rational design of drugs against trypanosomes, which are the causative agents of sleeping sickness in man, and nagana in cattle.

These analyses have, in particular, given further insights into i) the interactions of substrate analogues bound at the active site of TIM, ii) the conformational changes induced by binding of substrate analogues to TIM, iii) the effect of crystal contacts on crystallographic binding studies, and iv) conserved structural features of TIM from evolutionarily distant sources.

Ligand binding and conformational changes in TIM.

Binding of substrate analogues to TIM leads to a major conformational change of loops 5, 6, and 7. Chapters 2, 3, 5, and 6 analyse different conformational states of trypanosomal TIM, and the extent to which these crystallographically determined states are dependent upon the crystalline environment. In chapter 2 are analysed the consequences of transferring a $P2_12_12_1$ crystal of trypanosomal TIM grown from 2.4 M ammonium sulphate into a sulphate free solution. It was observed that this transfer lead to loss of the bound sulphate ion from the active site of subunit-2 of this crystal form, and to a conformational change of loop-6 (the "flexible loop") of that subunit from an "almost closed" to an "open" conformation. This movement of up to 7 Å in CA position could be discovered automatically using a crystallographically restrained molecular dynamics simulation. The resulting loop conformation was the first observation of a defined open loop conformation in the absence of stabilising crystal contacts. This conformation was seen to resemble closely the open loop conformation observed in subunit-1 of the same crystal form, where it is stabilised by crystal contacts. The movement of loop-6 was seen to involve residues 167-180. Sequential fragment superposition identified hinge regions at the start and end of this range, with the tip of the loop having the same conformation in both open and closed states. This conformation is stabilised by hydrogen bonds from the OG1 of Thr174 to main chain atoms of sequentially adjacent residues.

In chapter-3 were described the structures of trypanosomal TIM in complex with the substrate analogues glycerol-3-phosphate (G3P), 3-phosphoglycerate (3PGA), and 3-phosphonopropionate (3PP). These complexes were formed by soaking experiments using the $P2_12_12_1$ crystal form of trypanosomal TIM grown from ammonium sulphate. Ligand binding occurred only at the active site of subunit-2, i.e. that subunit to which sulphate is bound in the native structure. In the substrate analogue complexes, loop-6 was seen to have a new conformation, termed the "fully closed" conformation, differing by about 1 Å in CA position from the sulphate complex. This change was accompanied by a significant decrease in the atomic B-factors of the loop. The fully closed loop conformation was correlated with a 1 Å difference in the position of the phosphate moiety of substrate analogues when compared to the position of a bound sulphate ion. The side chain position of Glu167 was also seen to change upon binding of G3P or 3PP at the active site of TIM. This change gave rise to a positioning of the carboxyl group of this glutamate at a position (the "swung in" position), suitable for catalysing carbon-carbon proton transfer in the course of the catalytic cycle. In the absence of a substrate analogue, Glu167 was noted to interact with Ser96 (in the "swung out" position), and it was proposed that the availability of a hydrogen bonding partner for Glu167 in the ligand-free state modulates the overall free energy of binding of species along the TIM reaction coordinate. In addition to these changes, a rearrangement of loop-7 upon ligand binding was also noted. This change involved the flip of the peptide bond Gly212-Ser213, to allow the nitrogen of Ser213 to hydrogen bond to the phosphate group of the substrate analogues.

In chapter-5 were discussed crystallographic binding studies of trypanosomal TIM performed in a different crystal form, with space group $C2$, grown from PEG6000. This new crystal form had two monomers in the crystallographic asymmetric unit, each positioned on a crystallographic 2-fold axis, such that the constituent monomers of each molecular dimer were crystallographically equivalent. One of the molecules (molecule-1) of this new space group had loop-6 constrained by crystal contacts to maintain the open conformation, while the other (molecule-2) had a free loop-6. Transfer of a crystal of this space group to a solution containing 0.4 M ammonium sulphate gave rise to sulphate binding at the active sites of each molecule, but only at the active sites of molecule-2 was the corresponding electron density high. The sulphate ion was observed to bind 1 Å away from its binding site in the $P2_12_12_1$ crystal form, at a position slightly further outside the active site. Unlike the $P2_12_12_1$ crystal form, binding of sulphate at the active site did not give rise to closure of the flexible loop. Loop closure could, however, be brought about by binding the intermediate analogue phosphoglycolohydroxamate (PGH) to the active site of this subunit. Conformational changes similar to those observed in the $P2_12_12_1$ crystal form were also observed here for the free to ligand-bound transition. Even the subtle rearrangement of loop-5, which adapts to changes in side chain interactions of loop-5 with loop-6, was observed also in the new crystal form. Chapter-7 described a further crystal form of trypanosomal TIM with a single monomer in the crystallographic asymmetric unit. Comparison of the structure of this crystal form with the other two crystal forms showed that the combined coordinate error in the various trypanosomal TIM structures is probably less than 0.3 Å for atoms with low B-factor. In all ligand-free crystal structures, loop-6 was found to have a similar conformation, regardless of crystal contacts. The new crystal form was

also used to identify regions of trypanosomal TIM with unusual main chain conformation. This analysis identified the catalytic lysine, the start of loop-3, loop-6, loop-7, and loop-8. All of these regions are at the C-terminus of the β/α barrel.

In chapter-6 is described the complex of TIM with 2-(N-formyl-N-hydroxyamino) ethylphosphonate (IPP). This compound resembles PGH, except that the relative positions of the carbonyl and hydroxyamino functions are reversed. Thus, where PGH resembles substrate dihydroxyacetone phosphate, IPP resembles product glyceraldehyde-3-phosphate. The chemical differences between IPP and PGH gave rise to a difference in the positioning of the catalytic glutamate residue. It seems likely that this positional difference reflects a movement which occurs during the catalytic cycle.

Trypanosomal TIM in structure-based drug design.

One of the aims of crystallographic binding studies with trypanosomal TIM was to identify potential lead compounds which might be developed into selective inhibitors of TIM from *T. brucei*. For this purpose, the substrate analogues studied in chapter-3 were analysed with respect to accessible surface area. Unfortunately, these compounds were found to be almost completely enveloped in the active site of TIM, which is completely conserved between human and trypanosomes. Chapter-4 describes modelling attempts to predict the binding mode of 2-phosphoglyceric acid at the active site of trypanosomal TIM. These attempts succesfully predicted that 2-PGA would also bind in the active site in such a way as to prevent development away from the active site. Also described in chapter-4 is the crystallographic structure determination of the trypanosomal TIM/2-PGA complex.

The experiment giving rise to the sulphate-free structure described in chapter-2 was designed to identify the binding site of 1,4,6-trisulphono aminonaphthalene (N14), a selective inhibitor of trypanosomal TIM with an inhibition constant of 0.3 mM. The absence of electron density for this inhibitor in maps using data collected from a crystal soaked at 2mM suggested that the binding site might be blocked by crystal contacts. This gave rise to the search for new space groups described in chapters 5 and 7. Analysis of the regions involved in crystal contacts in all of the TIM space groups showed that, whereas packing might be very different, the protein regions involved in lattice contacts showed considerable conservation.

The TIM barrel fold.

In chapter 8 is described the crystallographic structure determination and characterisation of TIM from *E. coli* at 2.6 Å resolution. *E. coli* TIM is the first prokaryotic TIM for which a structure is available, and so extends the family of known TIM structures to cover vertebrates (chicken), protozoa (*T. brucei*), fungi (yeast), and prokaryotes (*E. coli*). The structure of this new TIM has been compared to previously observed TIMs. Notably, *E. coli* TIM lacks the cavity found in the core of the parallel β–barrel in other TIMs, due to the presence of a methionine residue (Met9) on β-strand 1 which points into the β–barrel, where all other TIMs of published sequence have a glycine or alanine residue. The nature of subunit-subunit interactions away from the

active site appears to be highly variable across evolution. By contrast, a high degree of conservation is observed for residues able to stabilise the N-terminii of the structural α-helices of the TIM barrel. The structure determination of *E. coli* TIM has also allowed modelling (described in chapter-9) of a β/α unit exchange mutant of TIM which has been produced by the group of Martial. This mutant is a catalytically active hybrid featuring β/α units 1-7 from *E.coli* TIM, and β/α unit 8 from chicken TIM. Matching of these units "in computro" suggests that the hybrid protein will have to adapt considerably to adopt the TIM barrel fold. The stability of the hybrid protein, despite numerous changes in side chain volume in packing regions, as well as a change in the α7–β8 loop evidences strongly the intrinsic stability of the $(\beta/\alpha)_8$ fold.