Triosephosphate Isomerase

The Structure of TIM

The amino acid sequences of TIMs, from both eukaryotic and prokaryotic sources have been determined. In all species, the enzyme is composed of two identical subunits, each consisting of roughly 250 amino acid residues. The two subunits interact closely and only the dimer is catalytically active (vide infra). The amino acid sequence has been determined by applying various degradation techniques on the enzyme but also by deduction from the gene sequence, including the human sequence. The molecular weight of each subunit is about 27,000D whereas the molecular weight of the dimer is about 53,000D. The variation in amino acid sequence of TIM of different species can be quite pronounced (figure 2.1). The figure shows a homology over 80% between human, rabbit muscle and Coelacanth TIMs, whereas the homology between T. brucei TIM and the enzymes of other species is about 50%.

However, the primary structure of the active site is highly conserved through evolution. The active site residues responsible for the catalytic activity are respectively: a lysine residue at position 13, a histidine at position 95 and a glutamic acid residue at position 165. These positions are not the exact number in the amino acid sequences. Due to the variation in sequence the exact number can be slightly different. In the trypanosomal enzyme, for instance, the glutamic acid is at position 167.

The cloning and sequencing of the gene has made site directed mutagenesis experiments possible. The mutagenesis has been applied to the catalytic active site residues.

Figure 2.1
Schematic representation of the percentage amino acid homology of TIMs of various species: T. brucei; Human; Rabbit muscle; Chicken; Coelacanth; Yeast; E. Coli; B. Stearothermophilus.
The glutamic acid residue number 167 has been much studied. Its function was first revealed by studies involving affinity labelling with irreversible covalently binding inhibitors (see page 35). The function of residue number 167 was made clearer by the replacement of the glutamic acid residue by an aspartic acid residue. 

This mutation moves the carboxylate function one methylene group i.e. about 1.5 Å away from the substrate. The result of this mutation is a 1500 fold decrease of the $k_{cat}$ of the mutant enzyme compared to the $k_{cat}$ of the wild type enzyme. Each subunit contains a flexible random coil loop composed of the residues 168-177. Pompliano et al. deleted four residues from this loop and studied the mutant enzyme. The specific catalytic activity of the purified mutant enzyme proved to be $10^5$ times lower than the wild type enzyme.

TIM belongs to the class of the α/β barrel enzymes. These enzymes share a common motive that they are composed of eight alternating α-helices and β-strands. The structural α/β-elements are coiled into a barrel so that the first β-strand is hydrogen bonded to the last, and that the inside contains an eight-stranded parallel β sheet surrounded by eight parallel α-helices. The active site is found at the C-terminal end of the β-barrel (figure 2.2).

A subunit of TIM not only consists of α-helices and β-strands, but there are also two random coiled loops that play a vital role in the functioning of the enzyme. The first loop (loop 3) connects α-helix 3 with β-strand 3 and consists of residues 70-80. This loop maintains the dimeric-structure of the enzyme and forms most of the inter-subunit contacts.

The other loop (loop 6) is very flexible and plays an important role in the reaction of the enzyme. As the substrate binds, this loop folds over the
active site, rendering it more hydrophobic. In this way the enzyme prevents the elimination of the phosphate group during the reaction which is an important side reaction of the isomerisation of DHAP to DGAP (see page 28).\textsuperscript{22,47,48}

The function of this flexible loop has been studied extensively. It was first described by Alber \textit{et al.}\textsuperscript{26} They noticed in crystallographic studies involving complexes of chicken TIM with inorganic phosphate and yeast TIM with DHAP, that the loop between residues 168 and 177 moved over a distance of 8Å and 10.4Å, respectively. Also the flexible loop of trypanosomal TIM has been studied by Wierenga \textit{et al.}\textsuperscript{31} using crystallographic methods with roughly the same results.

The interaction of the flexible loop with the substrate was also studied with molecular dynamics calculations by Brown and Kollman.\textsuperscript{27} In this study they also calculated the interaction of the flexible loop with an analogue of DHAP, \textit{i.e.} dihydroxyacetone sulphate (DHAS). The sulphate group in this substrate analogue has only one ionisation potential and hence, under physiological conditions, it has a single negative charge instead of a double negative charge as in DHAP in which the phosphate moiety has two ionisation states. They found that the loop did not move as much in the case of DHAS as in the case of DHAP. The reason for this was apparently that the single negative charge of DHAS had less attraction for the backbone amide hydrogens then the doubly charged DHAP.

In the previous paragraph, it has been mentioned that TIM is a dimer composed of two identical subunits (figure 2.3). Waley\textsuperscript{6} showed in a denaturation-refolding experiment that at a low concentration of the protein, the dimerisation became the rate-determining step for the reactivation of the protein. At higher concentrations the half life for reactivation becomes independent of concentration. This indicates that only the dimer is catalytically active and the monomers are not.

The dimeric structure is maintained by an interface loop (loop 3) between helix $\alpha_3$ and $\beta$-strand number 3. The amino acid sequence of this interface loop is conserved in all species.\textsuperscript{13} The pocket, in which the interface loop penetrates, is one wall of the active site of the other subunit. It appears that hydrogen bonding from the loop is essential for the integrity of the active site of the other monomer. Hence, the structure of the active site seems to depend on dimerisation.\textsuperscript{26}
**MECHANISM OF THE TIM REACTION**

In the preceding pages the main catalytic residues that are involved in the isomerisation reaction of TIM have already been mentioned. The most important residue in the active site is the catalytic base Glu167. The first indication of a single catalytic base was given by experiments in which the pH dependence of the forward and reverse TIM-reaction was studied.\(^4\) Other active site residues that are conserved across evolution are His95 and Lys13. These residues act as electrophiles polarising the carbonyl group of DHAP and thus rendering the hydrogen atoms on C\(_3\) more susceptible to abstraction by the active site base. The first indication of an electrophilic residue present in the active site was given by Webb and Knowles,\(^{14,15}\) in experiments involving the reduction of bound substrate by \[^3\text{H}\] labelled sodium borohydride. The reduction occurred approximately seven times faster on the enzyme surface than in free solution, so the enzyme must have catalysed the reduction. Webb and Knowles postulated the existence of an electrophile that increased the polarisation of the carbonyl group. Further evidence for an active site electrophile was provided by Belasco and Knowles.\(^6\) They studied the distortion of the substrate DHAP by TIM using Fourier transform infrared spectroscopy. In their experiments they observed a 19 cm\(^{-1}\) downward shift of the carbonyl stretching vibration. This shift is attributed to a polarisation of the carbonyl group by an active site electrophile more effective than water. Recently Komives \textit{et al.}\(^{17}\) demonstrated that His 95 is probably entirely responsible for the polarisation of the substrate carbonyl group. They repeated the FTIR experiments described above, for the H95Q and H95N\(*\) mutant TIMs in order to investigate whether His95 is the catalytic electrophile in the wild type enzyme. For the mutant enzymes, they found no shift in the substrate carbonyl stretching vibration. This indicates that the substrate carbonyls are not polarised by the mutant enzymes and consequently, that His95 must be the catalytic electrophile responsible for the polarisation of the carbonyl. It also rules out the assumption that Lys13 is involved in the polarisation of the carbonyl.

Recent crystallography\(^{62,63}\) and NMR studies\(^64\) on the complex of yeast TIM with the transition state analogue \textit{2-phosphoglycolohydroxamate} (2PGH, see also pages 33 and 34) revealed unambiguously that the histidine residue is the electrophile and it functions in an unexpected way. The proton transfer was studied by Bash \textit{et al.},\(^63\) who examined the energy along the reaction path of TIM with molecular dynamics calcula-
tions starting from the three-dimensional structure of the TIM-2PGH complex. The calculations suggest that His95 acts as an acid during the proton transfer from $O_1$ to $O_2$ of the ene-diolate to form an enediol. It must do so as an imidazole/imidazolate pair, which is highly unusual. The energy barrier for this proton transferring process is in the order of 13 kcal/mol. The proof of this new hypothesis was given by Lodi and Knowles. They studied wild type TIM and mutant TIMs, in which all histidine residues were deleted except for the active site histidine that had been isotopically enriched, with $^{13}$C- and $^{15}$N-NMR spectroscopy. Especially $^{15}$N-NMR has proven useful in the unambiguous demonstration that the imidazole ring of His95 is uncharged over the entire pH range of isomerase activity, between pH 5.5 and 9.9. This infers that the first $pK_a$ of His95 is below 4.5. This unusually low $pK_a$ value rules out that the imidazole ring is protonated during catalysis and hence, that a positively charged imidazolium cation is the electrophilic species in the active site. What the cause is for this unusually low $pK_a$ value is difficult to answer. The $^{15}$N-NMR reveals a highly deshielded $N_\delta_1$ in the His95 side chain. Lodi and Knowles argue that the active site $\alpha$-helix is responsible for a macro dipole with its positive end directed towards the His95 side chain. The imidazole side chain is edge-on to the helix with $N_\delta_1$ in the vicinity of the amino terminus of the helix (figure 2.4). This might account for the unusual deshielding of this nitrogen atom and the low $pK_a$ value of the imidazole ring. The NMR experiments on the enzyme in the presence of 2PGH show the presence of a strong hydrogen bond between $N_\varepsilon_2$ of His95 and the carbonyl of the bound inhibitor. These findings indicate the mechanism depicted in figure 2.5.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure24.png}
\caption{Two views of His95 and the active site helix ($\alpha_4$) consisting of residues 95-102 (from the coordinates of the wild-type yeast enzyme in the presence of 2PGH).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure25.png}
\caption{Putative mechanism for the isomerisation of dihydroxyacetone phosphate (DHAP) to D-glyceraldehyde-3-phosphate (DGAP) with residues Glu167 and His 95 of TIM acting as acid/base catalysts. Abstraction of a proton by the active site base first yields an enediolate intermediate that is strongly hydrogen bonded to the neutral imidazole side chain of His95. The imidazole proton is then transferred to the substrate with the transient formation of an enediol imidazolate pair. Abstraction of the proton of $O_1$ on the substrate by the imidazolate ion yields the isomeric enediolate, which collapses to give the product DGAP.}
\end{figure}
TIMs Side Reaction

Hence, the isomerisation of DHAP and DGAP proceeds through an enediol phosphate. This intermediate is very unstable and can suffer ω-elimination of the phosphate group to yield inorganic phosphate and methylglyoxal. TIM prevents this side reaction in two ways. Firstly by folding a flexible loop over the active site when the substrate binds. This renders the vicinity of the active site more hydrophobic and, consequently, diminishes the hydrolysis of the phosphate-ester bond. Secondly, stereo-electronic considerations suggest that elimination is least favoured if the orbital overlap between the enediol π-system and the C-O bond that links the phosphate group to C₃ is minimized. This conformation wherein the sp³-orbital of the C-O σ-bond is essentially perpendicular to the π-system is achieved by the enzyme by binding the substrate in its fully extended conformation. In this conformation of the transition state, the bridging oxygen of the phosphate ester group is planar with the enediolate moiety (see figure 2.6). Noble et al. observed that in the uncomplexed form, the substrate analogue 3PP (3-phosphonopropionic acid) is mostly extended, with O₁P, P, C₃, C₂, and C₁ trans-coplanar, but with the carboxyl group essentially perpendicular to this plane. This conformation minimizes the eclipsing interactions. In the enzyme-ligand complexes of both the 3PP T. brucei TIM complex and the 2PG yeast TIM complex, however, the carboxyl group is fixed into the plane of the rest of the molecule, resulting in an eclipsing interaction between the carboxyl group of 2PG and 3PP and the bridging group to the phosphate/phosphonate moiety (figure 2.6).

Figure 2.6
Above: In the left conformation of the transition state, the bridging oxygen of the phosphate ester group, sits below the plane of the enediolate moiety. In the right conformation of the transition state, the bridging oxygen of the phosphate ester group is planar with the enediolate moiety. This conformation minimizes the overlap between the sp³-orbital of C₃ and the π-orbital system and phosphate elimination is disfavoured. Below: In the left hand conformation 2PG has minimised the eclipsing interactions. The right hand conformation represents the one induced by TIM. The carboxyl group is forced in a conformation in which it is essentially coplanar with the rest of the molecule. This conformation is more strained due to eclipsing interactions of the oxygens with the aliphatic chain.
Inhibitors of TIM

Introduction

Generally, inhibitors can be divided in non-covalently binding or competitive inhibitors, and covalently binding or irreversible inhibitors. The irreversible inhibitors can be subdivided in "normal" irreversible binding inhibitors and irreversible mechanism based (IMB) inhibitors or suicide inhibitors. The irreversible inhibitors have been applied to investigate which active site residues are responsible for the reaction. The non-covalently binding inhibitors give more information about the way the enzyme functions. This group of inhibitors can be subdivided in substrate analogues and transition state analogues. Most of the reactions catalysed by enzymes are equilibrium reactions, the enzyme will catalyse both the forward and the reverse reaction. This means that the product of the reaction is also a substrate for the reverse reaction. Substrate analogues are therefore compounds that resemble either the substrate of the enzyme or the product of the reaction catalysed by the enzyme.

Transition state analogues on the other hand are compounds that resemble the putative structure of one of the intermediates that are encountered during the conversion of the substrate to the product. In 1946, Pauling suggested that an enzyme is able to accelerate a chemical reaction by having evolved an active site that is exquisitely complementary to the transition state. That is, although an active site must bind the substrate first, it is in the transition state that the binding interactions with the enzyme are maximized.\textsuperscript{50}

Knowles and Albery\textsuperscript{51,52} have discussed the factors that increase enzyme efficiency, and this bears on the issue of transition state complementarity. Residues involved in uniform binding interact equally strong with all species along the reaction coordinate. The residues involved in differential binding discriminate between the substrate and the product and the residues involved in the catalysis of the elementary steps preferentially bind one of the transition states along the reaction coordinate. The highest transition state is lowered due to evolutionary pressure and with this caveat in mind an enzyme’s active site is probably most complementary to the transition state with the highest energy barrier along the reaction pathway. In other words, the enzyme’s grip on the substrate tightens as the complex progresses toward the highest transition state, relaxing later as the product is formed and released.\textsuperscript{53}

When the active site of the enzyme is relatively rigid, the transition states of the individual steps along the reaction coordinate must bear a close resemblance. Hence, the enzyme has no difficulty in binding them all and the active site exerts a strain on the substrate towards the highest energy intermediate and carries it over the energy barrier to the product. On the
other hand, when the enzyme is flexible and can undergo large conformational changes it can adapt to each species along the reaction pathway and stabilise it.

In the case of TIM, there is an intermediate situation. Upon substrate binding, an extensive conformational change takes place that stabilises the substrate along the reaction coordinate to prevent decomposition of the enediol transition state. However, in the area around the reaction centre only the active site base, \textit{i.e.} Glu167, moves and the rest of the active site behaves like a rigid tube. This means that not much strain is exerted, since the substrate and the product bear a very close resemblance.

Transition states are difficult to study because, in general, they are too short lived to be observed. Their average lifetime is in the order of picoseconds. Wolfenden recognised in 1969 that, if a stable compound could be made that resembled an activated intermediate in substrate transformation, such a transition state analogue should bind tightly to the enzyme.\textsuperscript{33,35,53} The mechanistic significance of the tight binding of transition state analogues is quite evident. The design of an inhibitor is based on a guess as to the mechanism of the reaction, or on various experiments that suggest what the mechanism might be. The tight binding of an analogue provides powerful support for the mechanism on which its design is based.\textsuperscript{50}

### Competitive Inhibitors

Substrate Analogues

There are quite a few examples of substrate analogues for TIM. One of the first examples of a substrate analogue was given by Wolfenden,\textsuperscript{33,35} \textit{i.e.} DL-glycerol-3-phosphate (G3P). Johnson and Wolfenden\textsuperscript{35} found a \(k_i\) of 0.15 mM for chicken TIM and 0.23 mM for rabbit muscle TIM. Also, the affinity for \textit{T. brucei} TIM was measured by Lambeir et al.\textsuperscript{36} They found an \(k_i\) of 0.61 mM which is comparable to the affinity for the substrate DHAP (\(k_i=0.66\) mM).

G3P protected human and rabbit muscle TIM in experiments involving denaturation of TIM with guanidinium chloride.\textsuperscript{37} Hartman\textsuperscript{44} observed that G3P had enough affinity for TIM to decrease the rate of inactivation by irreversibly binding haloacetol phosphate inhibitors.

Wolfenden studied the effect of the binding of G3P on the cell dimensions of crystalline chicken TIM and observed that the cell dimensions diminished when the inhibitor bound to the enzyme.\textsuperscript{35} This was the first indication of a conformational change of TIM upon binding of a ligand. Jones and Waley\textsuperscript{38} extended the study of Johnson and Wolfenden\textsuperscript{35} by investigating the influence of pH on the binding of the transition state analogue 2-phosphoglycollate (2PG) and DL-G3P. They measured the change of the UV-spectrum as a function of the pH. The spectrum of the G3P-TIM complex changed progressively at low pH at 294 nm and 288 nm. An analysis of the relative positions of the aromatic residues in the scale...
bound and the unbound enzyme indicates that the movement of the flexible loop (loop 6, figure 2.2) affects interactions among Tyr164, Trp168, and Tyr208. These residues are probably the cause of the changes in the UV spectrum.

Between pH 6.5 and 8.5 the binding was independent of pH. The binding constant was about 0.86 mM. The affinity for TIM falls when the pH drops below 6, which is tantamount to saying that binding of the monoanion, the phosphate group carrying one proton, to the enzyme is markedly disfavoured.

Campbell et al. investigated the binding of G3P with $^{31}$P-NMR experiments. They observed that only the D-enantiomer binds to TIM.

The crystal structure of the complex of G3P and T. brucei TIM was solved by Noble et al.

Substrate analogues with a very close resemblance to DHAP and DGAP are the phosphonate analogues, i.e. 1-hydroxy-4-phosphonobuta-2-one and 2-hydroxy-4-phosphonobutanal, respectively. These analogues have been used to study the pH dependence on the binding of inhibitors. Dixon and Sparkes observed that the phosphonate analogue of DHAP was neither a substrate for chicken muscle TIM nor was it isomerised by TIM from Bacillus Stearothermophilus. The authors had no explanation for this absence of TIM activity on these analogues. However, Belasco et al. did observe that TIM catalysed the isomerisation of the phosphonate analogue of DGAP, i.e. 2-hydroxy-4-phosphono-butanal. This requires that the reverse reaction also proceeds and hence, that the phosphonate analogue of DHAP is also digested (see Intermezzo I: Binding of Phosphonates). The authors explained this discrepancy as follows:

1) the enzyme catalysed isomerisation of the phosphonate analogue of DGAP is some 800-fold slower than that of the natural substrate.
2) the equilibrium of the isomerisation lies predominantly toward the ketone and hence,
3) the enzyme assay is studied in the thermodynamically uphill direction, which is comparatively more difficult to perform.

Belasco et al. also studied another substrate analogue, i.e. dihydroxy-acetone sulphate (DHAS). They observed that this analogue is not a substrate for TIM. The reason for this is apparently the single negative charge of the sulphate group. As mentioned in one of the previous paragraphs (page 25), the reason for this is that the flexible loop does not fold effectively over the active site when a monoanionic substrate is present. This is also concluded from their pH study on the isomerisation of the phosphonate analogue of DGAP (vide supra).

Another obvious substrate analogue is the L-enantiomer of DGAP, the product of TIM. This analogue was studied by Richard. His objective...
The reason for the poor results with the phosphonate substrates seems to be a binding problem. This was also concluded by Dixon and Sparkes\textsuperscript{45} when they commented on the binding of the phosphonate analogue of 2PG, \textit{i.e.} 3PP.

![3PP](image1)

The affinity for TIM of the latter is some thousand fold lower than the affinity of 2PG. The apparent reason seems to be the absence of a hydrogen bond with the oxygen of the phosphate monoester. However, recent crystallographic studies by Noble \textit{et al.}\textsuperscript{40} revealed no interaction with this oxygen.

Lolis and Petsko\textsuperscript{58} reason that the specificity of TIM for phosphates may have little to do with hydrogen bonding. Rather this specificity depends on the preference of methylene groups for the staggered conformation. TIM forces the phosphonate inhibitors in an eclipsed conformation (see page 28), hence inducing steric hindrance between the phosphonate oxygens and the methylene hydrogens on the bridging methylene group C\textsubscript{3}, next to the phosphonate moiety. This might cost too much energy for an efficient binding to occur.

This type of steric hindrance is impossible in phosphate containing substrates and inhibitors. These compounds can therefore bind more efficiently.

\[
\text{DGAP} \quad \text{LGAP} \quad \text{HAP} \quad 3\text{-hydroxypropionaldehyde phosphate} \quad \text{2PGA} \quad \text{3PGA}
\]

was to study the racemisation of LGAP by TIM and the influence of deprotonation of LGAP prior to binding to the enzyme. The racemisation, \textit{i.e.} formation of DHAP and reverse reaction to DGAP, was only observed at high enzyme concentrations.

A comparison with the steady-state kinetic parameters for the reaction of DGAP and for LGAP shows an approximate decrease of $10^{6}$ in the first rate order rate constant $k_{\text{cat}}$ and $10^{9}$ in the second rate constant $k_{\text{cat}}/K_{M}$ for the reaction of the non-physiological substrate LGAP.

The reaction of TIM with the enediolate was not observed. Apparently this intermediate is too short lived to allow for a diffusion encounter with the enzyme. The $\beta$-elimination reaction to inorganic phosphate and methylglyoxal is faster than the isomerisation (see also page 28).

Other non physiological substrates that have been studied are the deoxy analogues of DHAP and DGAP, \textit{i.e.}, hydroxyacetone phosphate (HAP) and 3-hydroxypropionaldehyde phosphate respectively. The latter is, however, too unstable in solution to be studied at the pH and temperature necessary for the study with TIM. The proton exchange of these substrates with deuterated and tritiated water has been studied by Putman \textit{et al.}\textsuperscript{49} in 1972. They demonstrated that TIM catalysed the exchange of methyl protons of HAP with $D_{2}O$. The exchange rate, though not determined exactly, is about $10^{4}$-fold slower than for the natural substrate.

Finally, 2PGA and 3PGA also fall into the category of substrate analogues. Their affinity for trypanosomal TIM have been determined by Lambeir \textit{et al.}\textsuperscript{36} The racemic mixture of 3PGA had a $K_{i}$ of 1.3 mM, which is comparable to the affinity of the enzyme for the DHAP. The R-enantiomer is the more active species in the racemic mixture, since the L-
enantiomer of 3PGA, had a $K_i$ of 6.2 mM. This was confirmed by Noble et al.,\textsuperscript{40-42} who solved the three-dimensional structure of both the complexes of 2PGA and 3PGA with \textit{T. brucei} TIM. The racemic mixture of 2PGA had a $K_i$ of 6.9 mM. Steric interactions have been revealed in the aforementioned three-dimensional structure of the 2PGA-TIM complex. This is probably due to the fact that 2PGA is a rather bulky analogue of the substrate, which only fits with difficulty in the narrow active site.

In the case of TIM two transition state analogues are known: 2-phosphoglycollate (2PG)\textsuperscript{33-35} and 2-phosphoglycolohydroxamate (figure 2.7).\textsuperscript{56,61} 2PG is one of the first transition state analogues to be designed.\textsuperscript{33} The design of these inhibitors is based on the assumption that the transition state of the isomerisation of DHAP to DGAP is an enediol or enediolate structure.

2PG was first described by Wolfenden in 1969.\textsuperscript{33-35} It was described as a transition state analogue which bears a strong resemblance to the enediolate transition state both in charge distribution and stereochemistry, \textit{i.e.} the enediolate is a flat structure with a negative charge and this is mimicked by the carboxylate function of 2PG (figure 2.7). The inhibitory power is sensitive to the state of ionisation of an enzyme residue with an apparent $pK_a$ of about 7.35. Below this pH the inhibitor seems to dissociate.\textsuperscript{34} Wolfenden concludes, from the fact that kinetic and binding constants are dependent on pH, that 2PG is a transition state analogue.

2PG protected TIM against denaturation in Tris-HCl buffer at 67° C almost completely. It also protected against denaturation of human and rabbit muscle TIM by guanidine hydrochloride.\textsuperscript{37} It was much more effective in doing this than G3P.

The protection against heat inactivation suggests conformational changes upon binding of the inhibitor. This is also illustrated by the change of the UV-spectrum. The change is most marked in the region of the tyrosine and tryptophan absorptions (see also page 30).\textsuperscript{35} Moreover, Johnson and Wolfenden observed a strong contraction of chicken TIM crystals upon binding of 2PG. This change was larger than the one they observed upon binding of G3P.\textsuperscript{35} They interpreted this contraction of the unit cells as a conformational change of the protein.

Also protection against inactivation of irreversible inhibitors was observed,\textsuperscript{35} \textit{e.g.} the inactivation of TIM by glycidol-phosphate (\textit{vide infra}) occurred much slower in the presence of 2PG. This applies also to the inactivation of 1-bromo-3-hydroxyacetone phosphate (see page 35).\textsuperscript{57} Campbell \textit{et al.}\textsuperscript{59} investigated the interaction of 2PG with rabbit muscle and chicken muscle TIM, with $^{13}$C and $^{31}$P-NMR spectroscopy. They concluded that the bound form of 2PG is a tri-anion, \textit{i.e.} the carboxylic acid group is fully ionised upon binding to the enzyme. This means that
the enzyme is protonated in the process of binding this inhibitor. This observation is in accordance with the hypothesis that the transition state of the isomerisation reaction, catalysed by TIM, is an ionised species *i.e.* an enediolate ion. The authors also mentioned 2-phosphoglycolic acid amide as a very weak inhibitor. This appears to be understandable in view of its reluctance to form a comparable tri-anion.

Recently, the crystal structure of the complex of 2PG with yeast TIM has been published.\(^5\) In order to understand which residue is protonated upon binding of 2PG, the structure was investigated. Only the catalytic base, *i.e.* Glu167, seemed a plausible candidate. This residue changes from being hydrogen bonded to Ser96 to being hydrogen bonded to the carboxylate oxygen of 2PG. During binding the residue moves 2Å.

According to Lolis and Petsko,\(^5\) the origin of the tight binding of 2PG to TIM is mostly due to electrostatic interactions between the inhibitor and the enzyme, particularly His95 and Glu167. The dependence of \(K_i\) to ionic strength and the fact that the affinity of 2PG is higher at a lower pH indicates that electrostatic interactions are important for binding. Lolis and Petsko deduced that 2.5 kcal of the binding energy can be attributed to charge redistribution during the course of catalysis.

The inhibition constant of 2PG for trypanosomal TIM was determined by Lambeir *et. al.*,\(^3\) who found a value of 2.7 \(\mu\)M at pH 7.6.

2PGH is always ascribed to Collins,\(^5\) but in fact Lewis and Lowe\(^6\) published the inhibition of chicken TIM by this transition state analogue earlier than Collins. Lewis and Lowe already mentioned in their publication that the hydroxamic acid group is an excellent bioisostere of the enediolate transition state of the reaction of TIM (see figure 2.7). The partial double bond character of the amide bond in 2PGH and the preferred cisoid conformation of hydroxamic acids in non-polar solvents, seems to perfectly mimic the double bond, and the cisoid conformation in the transition state. 2PGH has a \(K_i\) of 4\(\mu\)M which corresponds to a 155 fold higher affinity than that of the substrate.\(^5\)

Collins made the first detailed study of the pH dependence of the inhibition of TIM by 2PGH and found that the \(K_i\) of 2PGH for rabbit muscle TIM did not vary between pH 7.5 and 8.5. Collins concluded from this that the hydroxamic acid moiety of the inhibitor binds in its neutral form to the enzyme because the pK\(_a\) of a hydroxamic acid group is about 9.5. However, he mentions that it is feasible that the catalytic base of the enzyme may abstract the nitrogen bound proton and form an internally hydrogen bonded structure analogous to the enolate structure of the transition state. Alternatively, Collins postulated that the neutral hydroxamic acid moiety may not be ionised, and the inhibitor may bind tightly largely because of its planar structure.
Recently, Davenport et al. described the high resolution structure of the complex of 2PGH and yeast TIM. They described the movement of the active site base Glu167. The active site is tightly packed with the inhibitor. From steric considerations the presence of water molecules can be ruled out. They are also not present according to the electron density maps, though the presence of disordered water molecules, which cannot be seen in the electron density maps, cannot be ruled out. This non aqueous environment enhances the basicity of the glutamic acid carboxylate side chain.

The active site electrophiles all form contacts with the oxygens of the hydroxamate moiety of the inhibitor. Lys12 interacts closely with the carbonyl oxygen of the inhibitor. The $N_{\varepsilon2}$ of His95 is positioned midway between the two hydroxamate oxygens. In this position it is possible that it mediates the transfer of the hydrogen from $O_1$ to $O_2$ in the isomerisation of DHAP. From the electron density it can be concluded that the His95 side chain is neutral in the complex with 2PGH and hence also in the enzyme-substrate complex (see also page 27).

The three-dimensional structure of the complex of 2PGH with trypanosomal TIM has been elucidated by Noble. This structure showed a 0.3Å rms coordinate difference for all 2PGH atoms when superimposed on the X-ray structure of 2PGH with yeast TIM.

Affinity labelling was one of the first tools used in the investigation of TIM to study the composition of the active site. The irreversible inhibitors used in these investigations were halo-ketone and epoxide containing compounds. Both types of irreversible inhibitors form an ester linkage with the active site base Glu167.

In 1968 Hartman reported that 1-iodo-3-hydroxyacetone phosphate ($X=I$) was covalently linked to the active site TIM and totally abolished enzymatic activity with the incorporation of 1.7 equivalents per mole of enzyme. Initially, Hartman thought that the modification took place via sulfhydryl groups on the enzyme. However, in 1971 a more detailed study on various haloacetol phosphates was reported. The residue modified by the inhibitor is shown actually to be a glutamic acid. The formation of an ester linkage occurs upon the binding of the haloacetol inhibitors, which is indicated by the formation of an hydroxamate when the modified enzyme was treated with hydroxylamine. A subsequent Lossen rearrangement of the resulting hydroxamate yielded 1,4-diaminobutyric acid, indicating that a glutamic acid residue formed an ester linkage with the inhibitor.

The pseudo first order kinetics of the loss of activity at large excess of inhibitor implicated that only one residue was modified. The observation that competitive inhibitors i.e. G3P and 2PG, protected the enzyme.
against inactivation indicated that the modified glutamic acid residue was located in the active site. The kinetic data indicated competition of both inhibitors for the same site.

The extremely high rate of inactivation of the haloacetol phosphates and the fact that a thousand fold excess of haloacetol phosphate did not react with free glutamic acid in solution at elevated temperatures and higher pH, indicates that the glutamic acid residue in the active site of TIM is highly reactive.

Although iodo-, bromo-, and chloroacetol phosphates react with the same residue, the second-order rate constants differ significantly. Usually the relative rates of displacement of a halogen in reactions with a $S_N2$-mechanism is $I>Br>Cl$. However, in this particular reaction the $k_{2nd}$ for iodoacetol phosphate is one tenth of the inactivation of chloro- and bromoacetol phosphate. Hence the inhibitor with the largest inherent reactivity is the slowest binding inhibitor. A reasonable explanation for this is that the large iodine atom has much difficulty in penetrating the narrow active site whereas the chlorine and bromine containing inhibitors can penetrate much easier.

A $^{31}$P-NMR study has been published in which the interaction of chloroacetol phosphate with TIM was studied. It turned out that there were two conformations of the enzyme: an unstable one and a stable one. The unstable conformation could not be regenerated when the inhibited enzyme was denatured with increasing amounts of guanidinium chloride and subsequently refolded. After this denaturation-refolding process one resonance (i.e. one conformation), the stable one, remained. The authors suggest that the two resonances are due to an open and a closed conformation of the flexible loop.

Another example of an haloacetol containing inhibitor is the sulphate analogue of chloroacetol phosphate. Chloroacetol sulphate also inactivates TIM by the selective esterification of Glu167. However, it reacts dramatically much slower than the phosphate containing analogue. This is in perfect accord with the fact that dihydroxyacetone sulphate is not a substrate for TIM (see page 31). The fact that a reaction takes place with this inhibitor suggests that the carboxylate anion of Glu167 has an extraordinary high nucleophilicity. The affinity for the active site of TIM of this inhibitor is very low so when a molecule enters the active site it also immediately reacts with the active site base.

Chloroacetol sulphate was studied in order to determine the $pK_a$ of the active site glutamic acid residue. The $pK_a$ value of the carboxyl group of the active site, as calculated from the pH dependence of the inactivation of yeast TIM, is approximately 3.9, and not about 6.0 as reported earlier. The changes in the apparent affinity of the enzyme for phos-
phorylated ligands near pH 6 are probably due to changes in the ionisation state of these ligands.

In 1975 Silverman et al. determined the inhibition of TIM by 1-fluoro-3-hydroxyacetone phosphate (X=F) and found it to be a comparatively weak irreversible inhibitor. Apparently, the poor leaving group character of the fluorine atom is the reason for its low reactivity.

In 1975 Silverman et al. determined the inhibition of TIM by 1-fluoro-3-hydroxyacetone phosphate (X=F) and found it to be a comparatively weak irreversible inhibitor. Apparently, the poor leaving group character of the fluorine atom is the reason for its low reactivity.

In 1969, Rose and O'Connell published on the inactivation of rabbit muscle TIM by glycidol phosphate. This inhibitor was designed to be an alkylating agent which resembled the planar structure of the enediolate transition state of the TIM catalysed reaction. They found an inactivation of the enzyme that was first order in active enzyme present leading to an 89% inactivation over 80 min. The formation of an ester was indicated by the liberation of radioactivity upon the treatment of $[^{32}\text{P}]$-labelled enzyme with alkali and hydroxylamine. The inactivation is quite specific since only one radioactive spot was observed after tryptic digestion of the labelled enzyme. This indicates that only one residue is labelled by the inhibitor.

In 1971 Miller and Waley published a more extended study of this inhibitor. They found that the active site base Glu167 is the site of attachment, as in the case of the haloacetol inhibitors. The ester that is formed is the ester derived from G3P. Miller and Waley also observed the inactivation of TIM by a mixture of glycidol (2,3-epoxypropanol) and inorganic phosphate.

The steric requirements for the inhibition are very strict. The enzyme is not inactivated irreversibly by 1,2-epoxypropyl phosphonic acid, which is found to be a competitive inhibitor.

Also here, the extreme reactivity of the Glu167 is noteworthy. Miller and Waley suggested that a hydrogen bond donating group might be present. Later it was discovered that His95 is this residue. Also the possibility that the glutamic acid group is unsolvated was suggested by the authors as an explanation of the high reactivity. These possibilities are receiving stronger support from various investigations.

**Design of New, Potential Inhibitors for T. brucei TIM**

The most important conclusion that can be drawn from the data in the literature, is that TIM is an enzyme that has evolved to almost perfection. For the current prevailing substrate concentrations the enzyme has reached a state of evolution in which little can be improved. This has resulted in a highly conserved active site in terms of primary and tertiary structure. About 85% of the residues within 10 Å of the active site of human, chicken and trypanosomal TIM are identical. Consequently, an in-
hibitor aimed at the active site of trypanosomal TIM is also an inhibitor with comparable affinity for human or chicken TIM. Hence, drug selectivity has to be found outside the active site. The residues which may be exploited for drug selectivity must be solvent accessible. The closest sequence dissimilarity that meets this requirement, is found some 10Å away from the centre of the active site at the sequence Ala100-Tyr101-Tyr102. This sequence will be aimed at in the present structure activity relationship (SAR) study.
What properties should the new inhibitors have? The inhibitors described in the literature reveal some general requirements:

1: They should have a group with two negatively charged ionisation states so that the flexible loop can fold entirely over the active site.
2: This group should be constituted by a phosphorous acid-like function.
3: They should not have extensive branching due to the fact that the active site is quite narrow.
4: The transition state analogues have the highest affinity for the active site and, hence, the inhibitor should be a transition state analogue or;
5: alternatively, a haloketone reacts irreversibly with the catalytic base. This reaction is so specific that even single negatively charged inhibitors react with this residue though much slower than the doubly charged counterparts.
6: In order to reach the selectivity sequence 100-102 an extension towards this sequence is necessary. This branching should be as close as possible to the phosphorous acid moiety of the inhibitor.
7: Preferably, the inhibitors should not be phosphonates due to the unfavourable conformation the enzymes imposes on the inhibitor upon binding. This results in a considerable loss of affinity compared to the phosphate counterparts.

Some of these requirements are conflicting, e.g. the requirements 1 and 6. The double negative charge necessary for a good folding of the flexible loop precludes extension from the phosphate moiety. Extensions with mixed phosphate diesters are therefore not allowed due to the loss of one negative charge.
Also, requirements 6 and 7 are conflicting. When the extension should be as close as possible to the phosphorous acid like part of the inhibitor, phosphonic acids are the ideal isosteres of phosphoric acids. Phosphoramidic acids have never been tested as inhibitors of TIM. The nitrogen bound to the phosphorous acid group also presents a possibility for extension. The fact that the selectivity area should be reached from the
active site (requirement 6) is in conflict with the fact that extensive branching is forbidden (requirement 3).
The observation that chloroacetol sulphate did bind to TIM, involves that the loss of affinity due to a single negative charge can be compensated by another part of the inhibitor. Therefore, requirement 5 can compensate a violation of requirement 1.
These constraints make it very difficult to design a selective inhibitor with a high affinity for trypanosomal TIM. The margins are very narrow and it is an excellent challenge for the protein crystallography techniques which are being implemented more and more in modern drug design, in order to design new inhibitors with a more rational approach.

The literature provided starting points for the active site anchors for the inhibitors. The requirements for anchoring groups are:
- High affinity for the active site
- Possibilities to extend or branch the inhibitor
Transition state analogues described in the literature have a higher affinity for the active site than substrate analogues. Moreover, transition state analogues provide an attractive basis for drug design because, unlike ordinary substrate analogues that are expected to affect two or more enzymes involved in formation and breakdown of the substrate, transition state analogues tend to be characteristic for a single enzyme. Also, irreversibly binding halo-ketones (see section 4, chapter 6) provide a firm, i.e. covalent, binding. These can be used to improve the affinity of the inhibitor for the area around the active site. Though the inhibitor might have lost affinity for the active site by the extension, the halo-ketone part provides for an irreversible anchoring and the part of the inhibitor that was designed to bind outside the active site is brought in the vicinity of the binding site for which it was designed. In this way, the interactions of weakly binding inhibitors with the surface of the enzyme around the active site can be studied.
Halo-ketone inhibitors may be unsuitable as future drugs for African trypanosomiasis. Their high reactivity might also damage other enzymes and consequently, severe side effects can be expected.

The strategy of rational inhibitor design was initially based on the concept of bioisosterism. This concept entails the following: bioisosteres are groups or molecules which have chemical and physical similarities producing broadly similar biological properties. A large number of systematic modifications can be made in the process of developing an anti-metabolite. The following parameters can be considered when making a bioisosteric replacement:
1: size
2: shape (bond angles and hybridization)
New Inhibitors for T. brucei TIM

Initially, transition state analogues were taken as lead compounds. The transition state analogues described above (page 33) both contain a phosphate group. An obvious bioisosteric replacement would be the substitution of the phosphate ester oxygen by a methylene group.\textsuperscript{71,72} This replacement changes the inductive effect of the group next to the phosphorous acid group and consequently the pK\textsubscript{a} of this function. Therefore, phosphonate containing substrate analogues have been used in the study of the pH dependence of the kinetic parameters for TIM (see page 31).\textsuperscript{46} The shape of these bioisosteric inhibitors has practically not changed.

However, the preference of the methylene group for a staggered conformation raises the energy content of the inhibitor when it is bound to the enzyme.\textsuperscript{58} Consequently, the affinities of the phosphonates for the enzyme are considerably lower than the affinities of their phosphate counterparts (see page 32).\textsuperscript{45,46} This disadvantage has to be accepted, because the bioisosteric replacement of the phosphate-ester oxygen by a methylene group is the only feasible possibility to provide a branching facility next to the phosphonous acid function (figure 2.8; alternative 1). The oxygen atom does not have such a potential. Phosphonic acids are for these reasons a general theme of this thesis.

An important advantage of the replacement of the phosphate group by a phosphonate group is the stabilisation of the compound, because the phosphonates are not vulnerable to cleavage of the phosphonic acid moiety from the rest of the inhibitor, than the phosphates. Consequently, the

---

**Figure 2.8**

Inhibitors designed starting from data from the literature. The R-group is meant to extend towards the selectivity areas.
ω-elimination of the phosphate moiety, described earlier (page 28), is suppressed, which opens the possibility to design inhibitors that do not require that the flexible loop closes entirely. Hence, more space will become available for branching.

Another possibility for the branching of the inhibitor at the atom next to the phosphonous acid group is provided by a nitrogen atom (X=N, alternative 2). In chapter 4, the syntheses of several variations on alternative 2 are described.

Extending the transition state analogues from the phosphorous acid group by means of phosphorous esters (alternative 3), though obvious, is probably not a good alternative because the resulting inhibitor has only one ionisable group. The necessity of a dianionic phosphorous acid moiety for affinity for TIM has been described extensively when dealing with the substrate analogue dihydroxyacetone sulphate (see pages 25 and 31).

Requirement 5 provides for another type of anchoring which counteracts the loss of a negative charge in the phosphate/phosphonate head group. An extension on this group might therefore be feasible when a halo-ke tone is used as anchor for the active site (alternative 4).

**References**

18. E.A. Noltman, "The Enzymes", 6 (1972) 326
References

22 D.L. Pompliano, A. Peyman, J.R. Knowles, Biochemistry, 29 (1990), 3186
23 G.K. Farber, G.A. Petsko, TIBS, 15 (1990), 228
34 R.Wolfenden, Biochemistry, 9(17) (1970), 3404
41 M.E.M. Noble, Thesis:"X-ray Crystal -
lographic studies of Triosephosphate Isomerase", Groningen (1992), 80
42 MEM Noble, C.LMJ. Verlinde, H. Groendijk, K.H. Kalk, R.K. Wieren -
44 F.C. Hartman, Biochemistry, 10(1) (1971), 146
47 J.P. Richard, Biochemistry, 24(4) (1985), 949
50 E. Lolis, G.A. Petsko, Annu. Rev. Bio -
chem, 59 (1990), 597
53 R. Wolfenden, L. Frick, "Enzyme Me -
54 G.E. Lienhard, Science, 180 (1973), 149
<table>
<thead>
<tr>
<th>Reference Number</th>
<th>Author(s) and Details</th>
</tr>
</thead>
</table>