An approach to the rational design of new inhibitors for trypanosoma brucei
Witmans, Cornelis Jacobus

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Appendix 2

X-ray structure of the 4PBH TIM complex

Abstract: The structure of triosephosphate isomerase from *Trypanosoma brucei* complexed with the competitive inhibitor \(N\)-hydroxy-4-phosphonobutanamide was determined by X-ray crystallography to a resolution of 2.84Å. Full occupancy binding of the inhibitor is observed at only one of the active sites of the homodimeric enzyme where the flexible loop is locked in a completely open conformation by crystal contacts. There is evidence that the inhibitor also binds to the second active site of the enzyme, but with low occupancy. The hydroxamyl group of the inhibitor forms hydrogen bonds to the side chains of Asn11, Lys13 and His95, while each of its three methylene units is involved in non polar interactions with the side chain of the flexible loop residue Ile172.

Interactions between the hydroxamyl and the catalytic base Glu167 are absent. The binding of this phosphonate inhibitor exhibits three unusual features: (1) the flexible loop is open, in contrast with the binding mode observed in eight other complexes between triosephosphate isomerase and various phosphate and phosphonate compounds; (2) compared with these complexes the present structure reveals a 1.5Å shift of the anion-binding site; (3) this is the first phosphonate inhibitor which is not forced by the enzyme into an eclipsed conformation about the P-CH2 bond. The results are discussed with respect to an on-going drug design project aimed at the selective inhibition of glycolytic enzymes of *T. brucei*.

RESULTS

4PBH binding by subunit 1

The present crystal structure provides a clear view of stereochemistry of the 4PBH binding by the active site of subunit 1 of trypanosomal TIM, as can be judged from the fit of the ligand into the omit density (see figure A2.1). A more quantitative proof of the presence of the inhibitor is given by the signal of the individual atoms in the omit map (see Table A2.I): the phosphonate and the hydroxamyl moieties are well defined; only the central atom of the aliphatic chain C3, has weak density. Furthermore, the individual atomic \(B\) factors of 4PBH are quite acceptable (see Table A2.I). They can be compared with an overall mean \(B\) factor for all atoms of the protein molecule of 20Å². A further assessment of the reliability of the inhibitor model is the evaluation of the dihedral angles. The conformation of 4PBH is staggered about P-C4, antiperiplanar about C4-C3, antiperiplanar about C3-C2 and (-)-synclinal for C3-C2-C1-O, *i.e.* the ligand is in a strainless conformation.

All potential hydrogen bond interactions of 4PBH are summarised in figure A2.2. The phosphonate group is directly coordinated by the main-chain NH group of Gly235 and the Oγ of Ser213. All other hydrogen bonds between the phosphonate and the protein are water-mediated. Compared with other phosphate/phosphonate inhibitor complexes, where the inhibitor binds in the closed flexible loop environment, only the hydrogen bond with Gly235 and the water-mediated interaction with Lys13 are retained. It is of considerable interest to establish whether this altered binding mode leads to a different anion positioning. Therefore, a superposition was carried out of subunit 1 of the 4PBH complex and subunit 2 of the monohydrogen phosphate TIM complex which yielded a r.m.s. difference on Cα positions of 0.30Å. However, the P-positions of inorganic phosphate and 4PBH are 1.54Å apart.

*This appendix was taken from the article: Structure of the Complex between Trypanosomal Triosephosphate Isomerase and \(N\)-hydroxy-4-phosphonobutanamide: Binding at the Active Site despite an "Open" Flexible loop Conformation. *C.L.M.J. Verlinde, C. J. Witmans, T. Pijning, K.H. Kalk, W.G.J. Hol, M. Callens, F.R. Opperdoes*, *Protein Science*, 1 (1992), 1578
At the other end of 4PBH the hydroxamyl oxygens are in contact with three electrophiles: the $N_{\delta2}$ of Asn11, the $N_{\zeta}$ of Lys13 and the protonated $N_{\varepsilon2}$ of the neutral His95 imidazole (figure A2.2). In addition, a water molecule mediates an interaction between the carbonyl oxygen of the hydroxamyl and the $O_{\gamma}$ of Ser96. Whether the 3.6Å distance between the hydroxamyl nitrogen and the main chain carbonyl of Gly211 is indicative of a hydrogen bond is debatable. 4PBH makes also nonpolar interactions. Each of its three methylene groups is in contact with $C_{\delta1}$ of Ile172: the distances are 3.2, 3.7 and 3.4Å from respectively $C_2$, $C_3$ and $C_4$. At the present resolution, 2.84Å, it is not clear whether the 3.0Å between $C_2$ and the main chain carbonyl of Gly211 is a bad contact. It is worthwhile mentioning that the catalytic residue Glu167 is still in its "swung out" conformation, i.e.

<table>
<thead>
<tr>
<th>Atom</th>
<th>B</th>
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<tr>
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<tr>
<td>P</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
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<tr>
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<td>6</td>
</tr>
<tr>
<td>C1</td>
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<td>9</td>
</tr>
<tr>
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<tr>
<td>N</td>
<td>36</td>
<td>7</td>
</tr>
<tr>
<td>O(-N)</td>
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<td>6</td>
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Table A2.I
Atomic B factors (Å$^2$) of 4PBH after refinement together with the electron density levels (i.e. number of standard deviations above zero) in the omit electron density map.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Free</th>
<th>Complex</th>
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<td>Atomic van der Waals radii used in the calculations were:</td>
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<tr>
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<tr>
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<td>1.90Å</td>
</tr>
<tr>
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<td>1.90Å</td>
</tr>
<tr>
<td>C(H)</td>
<td>1.40Å</td>
<td>1.40Å</td>
</tr>
</tbody>
</table>

Table A2.II
Burial of molecular surface$^8$ of 4PBH upon complex formation with gTIM: surface of free and complexed 4PBH (Å$^2$).
\( \chi_1 \) is (-)-synclinal, and is therefore unable to participate in the ligand binding.

**4PBH binding by subunit 2**

The final difference Fourier map contoured at a 1.5 \( \sigma \) level (figure A2.3) suggests that 4PBH binds also to the active site of subunit 2. Interestingly, the electron density also indicates that the side chain of Glu467 adopts a "swung in" conformation upon 4PBH binding while there is no density for an alternative flexible loop conformation. In contrast to the binding of 4PBH to subunit 1, however, the ligand position is only partially occupied in the crystal. If atomic signal of the putative P atom, which is 4\( \sigma \), is compared with its counterpart in subunit 1 it leads to an estimated 0.25 occupancy. The low \( B \) factors of the Glu467 side chain (mean \( B \) value is 13.5Å\(^2\)) in the "swung out" conformation are also in accordance with a low occupancy for the alternative conformation. In view of the 2.84Å resolution limit no attempt was undertaken to build a 4PBH model into the density of subunit 2.

**Structural adaptation of TIM to ligand binding**

It is possible to examine whether the presence of 4PBH alters the structure of the enzyme by comparing the crystal structure of the present trypanosomal TIM complex with the structure of the isomorphous unligated TIM crystals.\(^1\) A least squares superposition using the 498 C\(_\alpha\)'s of the two structures yields a r.m.s. difference on C\(_\alpha\) atomic positions of 0.25Å. On the basis of this superposition not a single protein atom involved in the coordination of 4PBH at the active site of subunit 1, directly or water-mediated, reveals a difference in position of more than 0.61Å.

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**Figure A2.2**

Interactions between 4PBH and trypanosomal TIM. Potential hydrogen bonds are shown in dashed lines with the distances between donor and acceptor indicated in Angstroms.

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**Figure A2.3**

Stereoscopic view of the electron density at the putative 4PBH binding site in subunit 2 of TIM, using the same map which was used for figure A4.1. The map is contoured at 1.5 times its standard deviation, in contrast with the 3\( \sigma \) level of figure A4.1.
The presence of the ligand at that site has thus no detectable influence on the enzyme conformation. In contrast, at the active site of subunit 2 the low occupancy binding of 4PBH is accompanied by at least a change in the conformation of Glu467.

**DISCUSSION**

In the crystal, phosphate or phosphonate-derived inhibitors like glycerol-3-phosphate, 3-phosphoglyceraldehyde, 3-phosphoglycerate, 3-phosphonopropionate bind to the active site 2 of gTIM with a concurrent closing of the flexible loop of the enzyme. This closure also occurs with inorganic phosphate or sulphate. No binding has been observed with any of the aforementioned inhibitors in active site 1 of gTIM where the flexible loop is fixed in an open conformation by crystal contacts. In contrast, the present study shows that 4PBH binds in both active sites of gTIM while in each case the flexible loop is in an open conformation. Full occupancy binding occurs in active site 1 while the occupancy is 25% in subunit 2. In addition, the position of the phosphor atom of 4PBH differs by 1.5Å from that of phosphor seen in five other inhibitor complexes of T. brucei TIM with phosphates or phosphonates.

Related to the latter point is the question why the flexible loop does not close down upon 4PBH in subunit 2 as was observed with the other phosphate/phosphonate inhibitors. Assuming that the full occupancy binding model of 4PBH in subunit 1 is also valid for subunit 2 a hint is possibly provided by the different phosphorus/sulphur position in gTIM complexes with inorganic phosphate, inorganic sulphate and 4PBH. Inorganic phosphate is the deepest buried in the complex of the three ligands while the flexible loop is in an open conformation. Sulphate sits 0.9Å further to the outside of the active site with the flexible loop in the 'almost closed' conformation; it still makes a hydrogen bond to NH of the flexible loop residue Gly473. The phosphorus atom of 4PBH is yet another 0.6Å further from the active site thus making it very difficult to create a hydrogen bond between the phosphonate and Gly473, if the flexible loop would be closed. The question remains why the phosphonate of 4PBH adopts this other position. Most likely, this is merely caused by the size of the inhibitor. 4PBH is conceptually one methylene unit longer than the substrate. Nevertheless, inside the active site it still interacts with the same residues (Asn11, Lys13 and His95) responsible for binding the substrate and inhibitors like glycerol-3-phosphate without causing a conformational change of these residues. The extra spacer thus pushes the phosphonate further out of the active site, and is allowed to do this because the alternative anion binding mode possibility.

An intriguing question concerning the present crystal form is why 4PBH binding to subunit 1 is preferred over binding to subunit 2. This is more difficult to understand since the previous studies have shown that subunit 2 can be 'open' (without inhibitor), 'almost closed' (with sulphate), as well as 'fully closed' (with phosphates and phosphonates). Therefore, the two binding sites were compared in detail. A least-squares superposition using Cα's of subunit 1 and subunit 2 was carried out yielding a r.m.s. difference on Cα atomic positions of 0.40Å. After omitting the Oγ of Ser213/513, for which the electron density is heterogeneous in subunit 2, the r.m.s. difference for the 11 atoms directly involved in the binding of 4PBH was found to be 0.53Å. Whether such small differences in atomic coordination can explain the differential binding is unclear. It remains puzzling, however, that poorer inhibitors such as phosphate ($K_i = 5mM$) bind with full occupancy to subunit 2 while 4PBH, with $K_i$ of 0.33 mM, has an occupancy of only 25%. We have as yet no explanation for this observation.

It is instructive to compare the binding mode of 4PBH to T. brucei TIM with the binding of 2-phosphoglycolohydroxamate (2PGH) to yeast TIM. 2PGH is a true transition-state analogue, having one methylene unit less than 4PBH and the phosphonate replaced by phosphate. It exhibits a $K_i$ of 4μM, which corresponds only to an 82-fold increase in binding affinity in comparison with 4PBH. Yet the interactions of the two inhibitors with TIM are very different, and can be summarized as follows: (1) 2PGH binds in a closed flexible loop environment whereas the loop is in an open conformation about 4PBH. Thus, 2PGH occupies with its phosphonate group the "classical" phosphate binding site whereas 4PBH binds some 1.5Å further to the outside of the active site; (2) The hydroxamyl-protein interactions with Lys13 and His95 appear to be similar for both inhibitors. However, in the yeast TIM-2PGH...
complex the catalytic residue Glu165 is in a "swung-in" conformation ($\chi_1$ is (+)-synclinal) and has abstracted a proton from the hydroxamic acid function. As we do not observe a hydrogen bond between the N of 4PBH and Glu 167, which is in a "swung out" conformation, it is likely that 4PBH binds to TIM as a dianion instead of a trianion, according to Collins, the pK$_a$ of the hydroxamic acid is 9.5; (3) A further difference involves the non-polar interactions. Extensive interactions are made between Ile172 and the three methylene groups of 4PBH whereas 2PGH interacts only with the C$_\alpha$ of Gly232. Consequently, it is not inconceivable that in the 4PBH-TIM complex hydrophobic interactions compensate in part for a potentially poorer binding of the phosphate and hydroxamyl groups; (4) Internal strain of the inhibitors may also contribute to difference in affinity, as discussed by Lolis and Petsko when they compared pairs of phosphate and phosphonate inhibitors. 4PBH is in a staggered conformation about the P-C$_4$ bond. Hence, it does not have to pay the energetic price observed for the shorter phosphonate inhibitors like 3-phosphonoproprionate, which are forced into an eclipsed conformation by the enzyme. 2PGH, on the other hand, adopts an eclipsed conformation about the P-O ester-bond: it is 42° away from the staggered conformation in one subunit and 32° in the second one. In summary, the comparison of the two inhibitor complexes clearly shows that very similar inhibitors can have different binding modes. How this structural knowledge can be translated into relative binding affinities is not trivial, also because desolvation of the inhibitors has to be considered.

For the further development of inhibitors of trypanosomal TIM, this study shows that it is possible to follow a new line of attack, namely to go for inhibitors which bind to the active site and at the same time prevent the closure of the flexible loop. The shift of the phosphonate with respect with its "normal" position and the open loop environment offer the possibility to branch 4PBH a C$_4$ as can be seen from Table A4.II. In contrast with the inhibitors bound in a closed flexible loop environment a large part of that carbon atom remains exposed in the complex with the enzyme. Work is currently underway to explore how such branching could lead to selective gTIM inhibitors.

Acknowledgements
We enjoyed stimulating discussions with Drs. Rik Wierenga and Martin Noble of the EMBL in Heidelberg. This research was supported by a grant of the WHO/UNDP/World Bank Special Programme for Research and Training in Tropical Diseases, by a fellowship from Hoffmann-La Roche, Basle to C.L.M.J. Verlinde, and by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for Scientific Research (NWO). The coordinates of the TIM-4PBH complex have been submitted to the Brookhaven Protein Data Bank.

References

8. Molecular surface calculations were carried out using the program MS (M.L. Connolly, J. Appl. Crystallogr., 16 (1983), 548) for the gTIM-4PBH complex and its individual constituents. Extended atomic radii for protein atoms were taken (J.A. McCammon, P.G. Woylnes, M. Karplus, Biochem., 18 (1979), 927) and derived by analogy for 4PBH.