An approach to the rational design of new inhibitors for trypanosoma brucei

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 13-12-2019
Section

Appendices
APPENDIX 1

X-ray diffraction of the iso-3PPH T.brucei TIM complex

METHODS

Soaking conditions

Purified trypanosomal TIM \(^1\) crystals were grown from 2,4 M ammonium sulphate in MOPS pH 7.0 from a solution containing also 1 mM dithiothreitol and 1 mM sodium azide. Binding of the protein in these crystals was achieved by transferring them to a solution of the alternative precipitant polyethylene glycol 6000 (PEG 6000) in MOPS pH 7.0 containing 100 mM ammonium sulphate, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM sodium azide. From this solution, crystals were transferred to a similar solution of PEG 6000 containing 2.5 mM iso-3PPH in place of the ammonium sulphate. The protocol used in the transfer has been described previously. \(^2\) The crystals were soaked for at least 7 days after which they were mounted in glass capillaries and three dimensional datasets were collected.

Data collection and processing

The Groningen FAST system was used in conjunction with an Elliot GX21 rotating anode generator. The datacollection protocol was chosen with the help of the STRATEGY program, implemented by Dr. Ivan Vickovic as a sublevel of the MADNES \(^3\) package. Initial data reduction was done using the program MADNES , with profile fitting being carried out by the program XDS. \(^4\) Scaling and merging of reflections was completed by use of programs from the Groningen BIOMOL suite. The statistics for the data collected were as follows: space group P2\(_1\)2\(_1\)2\(_1\), Cell dimensions: a=112.39Å, b=97.38Å, c=46.67Å, max resolution: 2.5Å, 15694 unique reflections, completeness: 85.3%, Rmerge(formula)=5.3%.

Starting model and structure refinement

The starting protein model used was the trypanosomal TIM structure observed in the G3P-TIM complex, which had an R-factor of 14.7% for data between 15.0 and 2.2Å. \(^5\) This structure was chosen in preference to the higher resolution native structure (1.83Å) since the protein structure of TIM in substrate analogue complexes had previously been observed to be different from the protein structure in the native complex with sulphate.

For purpose of comparison, the reflection data derived from the complexes were linearly scaled to the data of the native crystals which had themselves been placed on an approximate absolute scale. Refinement was begun by means of rigid body least squares procedures using the TNT package \(^4\) against data between 6.0Å and 4.0Å leading to a drop in the R-factor for this resolution range of 22.1% to 21%. Subsequently SIGMAA weighted 2mFo-DFc and mFo-Fc electron density maps \(^6\) were examined on an Evans and Sutherland picture system using interactive graphics package FRODO. \(^7\) The difference electron density maps suggested that the ligand bound only at the active site of subunit-2 of the enzyme. Positive and negative features of the difference electron density in the complex suggested the movement of the active site residues Glu467 and Leu532. The difference electron density for the ligand was of poor quality, suggesting the need of further refinement of the structures.

In order not to bias the active site electron density towards any particular conformation of the ligand, the ligand was not included for the first part of the structure refinement. This refinement involved initially least squares refinement with the TNT package at gradually increasing levels of resolution. This proto
col was followed by a brief protocol of molecular dynamics (MDX) and energy refinement (EMX) using the GROMOS package with a similar protocol to that previously described for the refinement of three inhibitor complexes of trypanosomal TIM. After refinement, the structure was subjected to a further 10 cycles of TNT positional refinement, and then 5 cycles of TNT B-factor refinement before being used to phase calculation of further mFo-Fc and 2mFo-DFc maps. At this stage in the refinement, the R-factor of the complex was 17.3% (15.0Å to 2.5Å).

Water molecules were selected from peaks in the difference electron density above 4 times the standard deviation of the map, provided that their environment was chemically sensible. The atomic model of the inhibitor was also manually built into the difference electron density using the program FRODO. The model was then subjected to further cycles of TNT restrained least squares refinement of positional and thermal parameters, with ideal geometries defined in the TNT package from the atomic models derived from BIOGRAF minimisation of the structure. The refinement statistics were as follows: R-factor = 15.3% for the resolution range 15.0Å-2.5Å, 15693 reflections included; the model included 3778 protein atoms, 106 water molecules and 10 ligand atoms; rms difference from the ideal geometry: 0.012Å for covalent bonds lengths, 2.3° for bond angles. In the observed structure of the iso-3PPH complex, the hydroxamic acid conformation is cis-planar and hence the constraint we imposed on the cis-planarity of the hydroxamic acid function during the modelling experiments seems to be justified.

Ramachandran plots of the structure of the complex of iso-3PPH with trypanosomal TIM showed almost no residues lying in forbidden regions of phi-psi space, and Luzatti plots of the agreement with the crystallographic data suggested, that the rms coordinate error was less than 0.2Å. A further assessment of the quality of the final model is the quality of the density calculated for the ligand. The fit of iso-3PPH into the omit density is shown in figure A1.1. The conformation can clearly be deduced from the electron density. From this figure and from figure 3.4 (see page 51) it can be deduced that coordination of the phosphonate group is similar to that observed in previously determined trypanosomal TIM-inhibitors. This similarity even extends to the distribution of water mediated hydrogen bonds between the ligand and the protein. All the potential hydrogen bonding groups of the ligand have hydrogen bonding partners with the protein.

**Results**

There is a marked similarity between the interactions made by iso-3PPH with TIM and the interactions made by G3P with TIM. Both ligands form good hydrogen bonds with the catalytic residues Lys313, His395 and Glu467. This justifies the assumption we made in the a priori modelling of the ligands. The differences in interactions result from the difference in geometry of the G3P and iso-3PPH: the iso-hydroxamate group of iso-3PPH occupies a planar conformation, whereas the two hydroxyl groups of G3P are staggered with respect to each other (figure A1.3). The consequence of this in terms of protein interactions is to introduce a symmetry into the env -
environment of the hydroxyl and the carbonyl oxygens of iso-3PPH which is absent in G3P. This can be seen in figure 3.4 (page 51), where the two oxygens of iso-3PPH are seen to be symmetrically disposed relative to the catalytic histidine, with each oxygen receiving a further hydrogen bond from either Lys 313 or Asn 311. There is, however, asymmetry in the interactions of iso-3PPH with the catalytic glutamate which is seen to interact only with the hydroxyl oxygen of iso-3PPH. This is a further indication that the side chain of glutamate 467 is charged. Work of Campbell\(^1\) indicated that 2PG binds as a trianion (page 33). This means that the enzyme becomes protonated. The X-ray structure of the complex of yeast TIM with 2PG also indicates a hydrogen bond between the carboxylic acid moiety of the inhibitor and the side chain carboxylate of Glu-467. Lolis and Petsko\(^1\) think it to be reasonable that the side chain carboxylate group of Glu-467 becomes protonated since this is known to be a very strong interaction between two carboxylates. A similar interaction is observed

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**Figure A1.2** A: Polar contacts of iso-3PPH with trypanosomal TIM. B: Polar contacts of G3P with trypanosomal TIM.

These are the contacts less than 3.2 Å involving an N or an O of the ligand, and an N or an O of the protein or solvent. These interactions include potential hydrogen bonds, and the interactions of the catalytic glutamate (Glu167).

**Figure A1.3** Stereoscopic comparison of the crystallographically observed complexes of trypanosomal TIM: a superposition of the complex with iso-3PPH (open bonds) and the complex with G3P (thin lines). Crosses indicate crystallographically observed water molecules.
by Noble et al.\textsuperscript{13} in the structure of \textit{T. brucei} TIM with 3PP, the phosphonate analogue of 2PG.

In the yeast TIM 2PGH structure,\textsuperscript{12} the carboxylic acid on the side chain of Glu467 has two interactions with the hydroxamic acid moiety of the inhibitor. One hydrogen bond with the nitrogen atom and one with the hydroxyl group. This supports the assumption of Collins\textsuperscript{14} that the hydroxamic acid function might be deprotonated upon binding to the enzyme. In \textit{iso-3PPH} the hydrogen atom on the nitrogen is absent. A hydrogen bond between the carboxylic acid group of Glu-467 is observed only with the hydroxyl oxygen of the inhibitor. Also an indication of a hydrogen bond interaction with the carbonyl oxygen is absent. Hence, this is a strong support for a charged glutamate.

A further difference between the complex of TIM with G3P, and the complex of TIM with \textit{iso-3PPH} is that \textit{iso-3PPH} appears to make less unfavourable short contacts than does the ligand G3P. This supports the hypothesis that \textit{iso-3PPH} is an transition-state analogue. One limitation of the modelling protocol used in both cases was the assumption of a fixed protein scaffold to which the idealised molecules were fitted. To assess the validity of this assumption in this case, the protein structure of the observed enzyme ligand complex can be compared with the protein structure used in the modelling experiments, \textit{i.e.} with the protein structure observed in the G3P complex. Several subtle differences in protein conformation are observed (figure A1.3). Notably the catalytic glutamate has adopted a new conformation, further "swung in" than observed in the G3P complex. The direction of the carboxylate movement is towards the hydroxamate nitrogen of \textit{iso-3PPH}, which is at an equivalent position to a carbon atom of G3P or the substrate. The shift could be due to the previously discussed differences in conformation between G3P and \textit{iso-3PPH}, or could be due to different interactions between the glutamate and the secondary alcohol of G3P, compared with the hydroxamic acid group of \textit{iso-3PPH}.

Another difference in protein structure is a slight shift of Asn 311, which is able to form a hydrogen bond to \textit{iso-3PPH} which could not to G3P, due to the different conformation of G3P. The last significant change is the movement of Leu 532, which adopts a conformation sterically precluded in the complex of G3P by the O1-atom of the ligand.

### Discussion

The qualitative nature of the protein ligand interactions in the observed structure agrees well with the assumptions used in the modelling of the compounds prior to the structure determination of the \textit{iso-3PPH} complex with TIM. This is encouraging because it demonstrates that it is possible to expand upon the knowledge obtained from previously determined structures i.e. the validity of the rational drug design cycle is confirmed here.\textsuperscript{15} It has to be stated that the success in the prediction of the binding mode of the ligands studied here is also due to several particular conditions in this analysis: \textit{e.g.} we predicted the binding mode of 3PPH and \textit{iso-3PPH} starting from a ligand with a comparatively small difference in structure. We had a firmly established starting point being: the principle determinant of ligand binding to TIM is the well defined interaction of main chain nitrogens with the phosphate group. Finally the binding site of the ligands is almost entirely sealed off from the solvent, removing the complications of treatment of the solvent in energy calculations.

For the rational design of specific inhibitors of trypanosomal TIM, this study has produced at least one interesting observation, namely that the C\textsubscript{1} atom of \textit{iso-3PPH} has been shown to be surrounded by space in such a way as to allow branching. Analysis of this space compared to the van der Waals surface of the enzyme suggests that it lies in a suitable direction for branching away from the active site. Further analysis reveals that a branch leading from C\textsubscript{1} would necessarily displace a water molecule which has been found in the structures of all five trypanosomal TIM-inhibitor complexes solved so far.\textsuperscript{5} This water molecule forms interactions with the ligand phosphate and with the Lys-313 of the enzyme.
References