Structure-based protein engineering efforts with a monomeric TIM variant: the importance of a single point mutation for generating an active site with suitable binding properties

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A monomeric variant of triosephosphate isomerase (TIM) with a new engineered binding groove has been characterized further. In this variant (ml8bTIM), the phosphate binding loop had been shortened, causing the binding site to be much more extended. Here, it is reported that in the V233A variant of ml8bTIM (A-TIM), three important properties of the wild-type TIM active site have been restored: (i) the structural properties of loop-7, (ii) the binding site of a conserved water molecule between loop-7 and loop-8 and (iii) the binding site of the phosphate moiety. It is shown that the active site of A-TIM can bind TIM transition state analogs and suicide inhibitors competently. It is found that the active site geometry of the A-TIM complexes is less compact and more solvent exposed, as in wild-type TIM. This correlates with the observation that the catalytic efficiency of A-TIM for interconverting the TIM substrates is too low to be detected. It is also shown that the A-TIM active site can bind compounds which do not bind to wild-type TIM and which are completely different from the normal TIM substrate, like a citrate molecule. The binding of this citrate molecule is stabilized by hydrogen bonding interactions with the new binding groove.

Keywords: citrate/monomeric TIM/protein design/suicide inhibitor/transient state analog

Introduction

Harnessing the powerful catalytic properties of enzymes for tailor-made organic chemistry synthesis is one of the goals of many current enzyme engineering efforts (Koeller and Wong, 2001; Hult and Berglund, 2003). In many of these efforts, the chiral specificity of the non-natural enzyme is of key importance (Heinze et al., 2007). Two principally different approaches are being tried, being (i) changing substrate specificity of existing enzymes or (ii) grafting new catalytic activities on existing frameworks. The latter example includes, for example, the study of catalytic antibodies (Hanson et al., 2005) as well as the de novo genesis of a new function on an existing framework (Dwyer et al., 2004; Allert et al., 2007). In both cases, computational approaches (Bolon et al., 2002; Ashworth et al., 2006; Alvizo et al., 2007) and experimental approaches (Jurgens et al., 2000; Hsu et al., 2005; Woycechowsky et al., 2007) are used. The experimental approaches use random mutagenesis as part of directed evolution screening and selection methods (Bloom et al., 2005; Carbone and Arnold, 2007; Reetz et al., 2007). In structure-based directed evolution, these computational and experimental methods are combined (Dwyer et al., 2004).

In the studies, which are reported here, it is attempted to change eventually the substrate specificity of a wild-type enzyme, triosephosphate isomerase (E.C. 5.3.1.1.) (wtTIM), which is known to have a very narrow substrate specificity (Knowles, 1991). This enzyme is a glycolytic, dimeric enzyme, catalyzing the interconversion of dihydroxyacetone-phosphate (DHAP) and D-glyceraldehyde-3-phosphate (DGAP) (Fig. 1). The catalytic specificity of TIM is achieved by the side chains of an asparagine, lysine, histidine and glutamate. The latter residue is the catalytic base (Knowles, 1991). The substrate specificity comes from interactions of the phosphate group of the substrate with main chain atoms of three loops, being loop-6, loop-7 and loop-8. The presence of these phosphate binding loops provides TIM with a very narrow substrate specificity, as the only known substrates are DHAP and DGAP. The tight binding of the substrate apparently prevents the phosphate elimination side reaction, which easily occurs in simple non-enzymatic base catalysis (Richard, 1984; Pompliano et al., 1990), because the phosphate moiety is an easy leaving group. In this side-reaction phosphate and methylglyoxal are formed. The active site is at the dimer interface, but each of the catalytic residues as well as the residues of the binding pocket is from the same subunit. It has been shown that mutational changes in the dimer interface loop convert the dimer into a monomer, which is still a competent TIM, but with 1000-fold lower $k_{cat}$ and 10 times higher $K_m$, when compared with the wild-type dimer (Schliebs et al., 1997; Thanki et al., 1997). Extensive enzymological characterization of the competent monomeric TIM (ml1TIM) has shown that its turnover number is $\sim$1 s$^{-1}$ and the $K_m$ is $\sim$5 mM (Schliebs et al., 1996; Thanki et al., 1997). The crystallographic studies of these monomeric TIM variants (Borchert et al., 1995; Thanki et al., 1997) have also shown that the mode of binding of the substrate analogs is very similar as seen in wild-type TIM. In addition, these studies have shown a large structural plasticity of the active site loops, in particular in the unliganded forms (Borchert et al., 1995).

A common feature of wtTIM and monomeric TIM structures is the presence of an open and closed state. Typically, the open state is observed for the unliganded complex,
whereas the closed state is seen in the presence of bound ligand. The structural differences between the two states concern a large movement of the tip of loop-6 (7 Å) and a smaller but correlated movement of two peptide planes in loop-7 (Casteleijn et al., 2006). Also the side chain of the catalytic glutamate, at the beginning of loop-6, switches from a swung-out conformation to a swung-in conformation, which is essential for its catalytic role.

In order to introduce a different substrate specificity for ml1TIM, a mutagenesis experiment was carried out in which the phosphate binding loop-8 was shortened. The characterization of this new variant, ml8bTIM (Norledge et al., 2001), showed indeed a much wider and more extended binding pocket. However, the new variant was inactive; also the affinity for the transition state analog 2-phosphoglycollate (2PG) was lost (Norledge et al., 2001). The ultimate goal of the current studies is to restore the catalytic activity of this variant for compounds in which the phosphate moiety of the wild-type substrate is replaced by a completely different, more extended moiety. Such an enzyme would be an important tool for making chiral \( \alpha \)-hydroxy aldehydes, which are important intermediates in various synthetic pathways, for example, for the synthesis of ribose analogs (Mikhailopulo, 2007). Chiral \( \alpha \)-hydroxy aldehydes are difficult to make via standard synthetic organic chemistry protocols because of their high reactivity (Waszkuc et al., 1984; Kern and Spiteller, 1996).

In order to characterize the properties of the ml8bTIM variant better, further studies with ml8bTIM and a range of mutated variants have been carried out to probe its binding and catalytic properties in a systematic manner. Through these efforts, a new crystal form of ml8bTIM was found. This new crystal form diffracted to much higher resolution and provided key information, namely that in ml8bTIM the side chain of Val233, at the beginning of loop-8, has adopted a new conformation, which interferes, in a subtle way, with the binding properties of its active site. Subsequently the V233A variant of ml8bTIM was made and it was found that in this variant (A-TIM) the binding of the transition state analog 2PG is restored. The mode of binding was found to be very similar, but not identical, as seen in wtTIM. Furthermore, the crystallographic binding studies of this new variant with a wild-type suicide inhibitor, bromohydroxyacetone-phosphate (BHAP), confirm the special catalytic properties of the catalytic glutamate of the new variant.

**Fig. 1.** TIM catalyzes the interconversion of DHAP and DGAP. Binding studies have been done with citric acid (CA), 2PG (a transition state analog), BHAP (a suicide inhibitor) and G3P (a substrate analog). Chiral carbon atoms are marked by asterisk.

**Materials and methods**

**Mutagenesis, protein expression and purification**

Site-directed mutagenesis was performed by PCR using a QuickChange site-directed mutagenesis kit (Stratagene). The expression plasmid pET3a (Novagen) containing ml8bTIM (Norledge et al., 2001) was used as a template. The mutagenic primers used were:

- V233A sense (+) 5'-AGACGTCAACGGCTTCCTTGCT
- AGCCTGCTTAAG-3'
- V233A compl. (-) 5'-TTAACGCAC
- GCTAGCAAGGAAGCCGTTGACGTCTCG-3'.

The complete DNA sequence of the TIM gene including the mutations was verified by using sequencing with the DYEnamic ET terminator cycle sequence kit (GE Healthcare). *Escherichia coli* TOP10 cells (Invitrogen) were used as the host strain for plasmid production. Plasmids were then transformed to *E. coli* strain BL21 pLysS (Invitrogen) for protein production.

Expression and purification of the trypanosomal TIM (wtTIM), ml8bTIM and the V233A-variant of ml8bTIM (A-TIM) were carried out as previously described (Casteleijn et al., 2006). A-TIM was additionally purified by size-exclusion chromatography.

**Enzymatic assays**

Unless specifically mentioned, all commercial chemicals were used without further purification. BHAP was synthesized as previously described (Casteleijn et al., 2006). Protein concentrations were measured with a nanodrop spectrophotometer (Nanodrop) at OD\(_{280}\).

TIM activity for wtTIM was assayed at 25°C (Sun and Sampson, 1999). DGAP was purified by use of Dowex-50. Glycerocephosphate dehydrogenase (GDH) was used as a linker enzyme. Residual TIM activity in the GDH sample was inactivated by incubation with BHAP, as previously described (Sun and Sampson, 1999). The DGAP-assay mixture contained NADH (0.3 mM), GDH (0.04 mg ml\(^{-1}\)) and DGAP (0.14–3.5 mM) in 0.3 ml buffer 100 mM triethanolamine hydrochloride (TEA), 1 mM EDTA, 1 mM DTT, 1 mM NaN\(_3\), pH 7.6. The inhibitory properties of citrate were studied in the presence of 1 or 5 mM citric acid. In the case of the presence of citric acid, the pH of the reaction mixture was checked, and corrected with NaOH when necessary, before use. wtTIM (7.5 ng) was used to initiate the reaction. Initial rates were measured at each substrate concentration from the change in NADH absorbance at 340 nm with a Powerwave X microtiterplate reader (Bio-tek Instruments). Path length corrections were measured according to the manufacturer, and confirmed with a NADH solution (0.05 mM) using a spectrophotometer and a 1 cm
The TIM activity of A-TIM was also measured at 25°C. The A-TIM (0.17–0.35 mg ml⁻¹) was added to assay mixture [NADH (0.3 mM), GDH (0.04 mg ml⁻¹) and DGAP (10 mM) in 0.5 ml buffer 100 mM TEA, 1 mM EDTA, 1 mM DTT, 1 mM NaN₃, pH 7.6], and the reaction was followed during 6 min.

The stability measurements of A-TIM were done by recording circular dichroism (CD) melting curves with a Jasco J-715 spectropolarimeter with a pathlength of 1 mm by monitoring the ellipticity from 20°C to 60°C at 222 nm with a rate of 30°C per hour. Protein solutions (0.3 mg ml⁻¹) were either in 0.9 mM TEA, 1.2 mM NaCl, pH 7.5 (unliganded enzyme) or in 2 mM 2PG, 0.9 mM TEA, 1.2 mM NaCl, pH 7.5 (enzyme liganded with 2PG). The protein solutions contained also 0.05 mM DTT, 0.05 mM EDTA and 0.05 mM sodium azide and were degassed before the measurements.

Crystallography

Crystallization conditions were screened using the Factorial 1 screen (Zeelen et al., 1994) at +22°C. The hanging drop method with 2+2 μl drops was used for obtaining the ml8bTIM crystals and the sitting drop method with 1 μl drops was used for growing the A-TIM crystals. The well solutions used for the optimized crystallization experiments are presented in Table I.

For ml8bTIM 12 mg ml⁻¹ protein, 20 mM TEA/HCl pH 8, 1 mM DTT, 1 mM EDTA and 1 mM NaN₃ were used as the protein solution. Before freezing (Table I), the ml8bTIM crystal was moved into a 10 μl drop of well solution, containing additionally 2.5 mM 2-(N-formyl-N-hydroxy)-aminoethyl-phosphonate (IPP). IPP is a substrate analog used previously in wild-type TIM binding studies (Kursula et al., 2001). The crystal was soaked for 5 min in this drop and subsequently it was moved to a 10 μl drop of well solution containing also 5 mM IPP and 5% glycerol for 5 min, before being frozen in the cold nitrogen stream.

In case of A-TIM the protein solution contained 11 mg ml⁻¹ protein, 20 mM Tris/HCl pH 7, 100 mM NaCl, 1 mM DTT, 1 mM EDTA and 1 mM NaN₃. The crystal structure of A-TIM (the A-TIM-CA structure; CA refers to citrate which was found to be bound in the active site) was obtained using data of a frozen crystal, which, before freezing in the cold nitrogen stream, was quickly moved through a drop of 100% paraffin oil. Three additional data sets were collected of A-TIM crystals soaked in solutions with three different substrate analogs, being 2PG, BHAP and glycerol-3-phosphate (G3P). These soaked crystals were obtained from A-TIM crystals that were handled by repeating the following protocol seven times: (i) drop was covered by 20 μl of 100% paraffin oil, (ii) crystal was transferred into a new 5 μl drop of the well solution, but with citric acid replaced by 0.1 M MES pH 5.5 and with additionally 10 mM ligand present (freshly prepared) and (iii) incubation in a hanging drop setup for 4–12 h with 1 ml of the same solution (but without ligand) in the well. All A-TIM crystals, except the A-TIM-2PG crystal, were moved through a drop of 100% paraffin oil before freezing. For the A-TIM-2PG crystal, the last soaking step was repeated with 20% ethylene glycol included in the soaking solution, before flash-freezing in a cold nitrogen stream at 100 K.

The data set of ml8bTIM, collected at the home source, was obtained using a Nonius FR391 rotating anode X-ray generator, equipped with a Marresearch MAR345 image plate and Prophysics XRM-216 mirror system. The synchrotron data sets were collected at EMBL/DESY, Hamburg, Germany on beam-line X13 (A-TIM-2PG, A-TIM-BHAP and A-TIM-G3P) and ESRF, Grenoble, France on beamline ID 23-2 (A-TIM-CA). All data were processed using program XDS (version June 2006) (Kabsch, 1993) with the interface XDSi version 1.2 (Kursula, 2004). Programs F2MTZ and CAD from the CCP4 package (version 6.02) (Collaborative Computational Project, Number 4, 1994) were used to flag 5% of the observed structure factors for the free R-factor calculations. The essential data collection statistics of these structures are shown in Table II. The structures were solved using molecular replacement with program MOLREP version 7.3.01 (Vagin and Teplyakov, 1997) using the low resolution ml8bTIM (PDB entry 1DKW; Norledge et al., 2001) as a search model. In each of the structure determinations, the loop-6 residues were deleted from the search.

Table I. Overview of structures and crystallization conditions

<table>
<thead>
<tr>
<th></th>
<th>ml8bTIM</th>
<th>A-TIM-CA</th>
<th>A-TIM-2PG</th>
<th>A-TIM-BHAP</th>
<th>A-TIM-G3P</th>
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<tr>
<td>Crystallization well solution</td>
<td>0.1 M Tris/HCl pH 8.5, 1.9 M MgSO₄</td>
<td>20% PEG6000, 2.5% t-butanol, 0.1 M citric acid pH 5.5</td>
<td>20% PEG6000, 2.5% t-butanol, 0.1 M citric acid pH 5.5</td>
<td>20% PEG6000, 2.5% t-butanol, 0.1 M citric acid pH 5.5</td>
<td>20% PEG6000, 2.5% t-butanol, 0.1 M citric acid pH 5.5</td>
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<tr>
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<td>22</td>
<td></td>
<td></td>
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<td>Paraffin oil method</td>
<td>Cryoprotectant: 20% ethylene glycol in soak solution</td>
<td>Paraffin oil method</td>
<td>Paraffin oil method</td>
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<td>2</td>
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<tr>
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<td>2PG</td>
<td>BHAP</td>
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<td>BHAP</td>
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model and rebuilt in the new electron density maps. Also, waters, active site ligands (Table I; Fig. 2) and other solvent molecules were built from scratch in the respective electron density maps. The structures were refined with REFMAC5 version 5.3.0028 (Murshudov et al., 1997). The bond length and the bond angle of the covalent bond between BHAP and Glu167 were restrained by defining a special link (Vagin et al., 2004). NCS restraints were used in the REFMAC5 refinement protocol for the A-TIM-2PG, A-TIM-BHAP and A-TIM-G3P structures. The anisotropic motion of the sub-units was described with the TLS parameters as implemented in REFMAC5 (Murshudov et al., 1997) for A-TIM-CA, A-TIM-BHAP and A-TIM-G3P structures. Program Coot version 0.3.1 (Emsley and Cowtan, 2004) was used for the manual rebuilding of the structures, for adding waters and for checking the quality of the structures. The Molprobity method (Lovell et al., 2003) was used to analyze the Ramachandran plot. The refinement statistics of these structures are shown in Table II.

Structure analysis
There are three molecules per asymmetric unit in the ml8bTIM structure. Molecule B is used for the structure analysis. No ligand is bound in any of the three active sites of ml8bTIM. In the A-TIM crystals, there are two molecules per asymmetric unit. Molecule A is used for a description of the structural features of the A-TIM-CA and the A-TIM-G3P complexes. In the latter experiment, the citrate molecule has completely diffused out of the active site of molecule A, without being replaced by G3P and therefore this provides an image of the unliganded active site. The liganded active site geometry of the 2PG and BHAP complexes is best defined in molecule B of the respective structures. Other structures that have been used: 5TIM (A-subunit) (1.83 Å resolution) (Wierenga et al., 1991), 1N55 (0.83 Å resolution) (Kursula and Wierenga, 2003), 1ML1 (molecule G) (2.6 Å resolution) (Thanki et al., 1997) and 1DKW (molecule A) (2.7 Å resolution) (Norledge et al., 2001). 1ML1 is the structure of ml1TIM, complexed with 2PG. 1DKW is a low resolution structure of unliganded ml8bTIM (Norledge et al., 2001). 5TIM(A) is the high resolution structure of unliganded, open TIM and 1N55 is the best structure of a liganded, closed complex complexed with 2PG. In the latter structure, a double conformation is observed for the 2PG mode of binding, such that the acid moiety is either in an up-conformation (which is the predominant form) or in a down conformation. 5TIM and 1N55 are wild-type TIM structures of, respectively, trypanosomal and leishmania TIM. These TIM’s are close homologs (Williams et al., 1999).

Superpositions have been done with the 36 Cα-atoms of the eight β-strands, residues 7–11, 38–42, 61–64, 90–93,
Results and discussion

The high-resolution structure of ml8bTIM

The initial crystal structure of ml8bTIM (1DKW) showed that this protein has a much more extensive binding pocket as in wtTIM, as aimed for in its original design (Norledge et al., 2001). Nevertheless, the transition state analog of wtTIM 2PG was shown to have no measurable affinity. The available crystals of ml8bTIM diffracted to 2.7 Å resolution, which is not sufficient for a detailed structural analysis. Further crystallographic studies of ml8bTIM produced a second crystal form. This crystal form, which has three molecules per asymmetric unit, diffracts much better, to 1.9 Å resolution. In this crystal form, there are three molecules per crystallographic asymmetric unit. Each of these molecules adopts an open/unliganded conformation. This structure has been refined to R-factor/R-free of 16.6%/21.5% with good statistics for its geometry (Table II). The analysis of this structure showed interesting non-wild-type-like features for loop-7. In each of the molecules, the side chain of Val214 of loop-7 has moved away from loop-8. In molecule A, it concerns only a small movement of residues 213 till 217, like in the previous ml8bTIM structure (1DKW), whereas in molecules B and C, the movement is more extensive (Fig. 3). Careful inspection of these structures and comparisons with the wild-type TIM structure (Fig. 3) explains this rearrangement of loop-7, which appears to be due to the outward movement (toward bulk solvent, away from the protein interior) of the side chain of Val233 of loop-8 such that it clashes with the wtTIM position of the side chain of Val214 of loop-7. Consequently Val214 shifts to a new position, causing a rearrangement of loop-7. Loop-7 is a key component of the competent active site of TIM, in particular for generating a proper binding site for the phosphate moiety of the substrate (Casteleijn et al., 2006). This outward movement of the Val233 side chain is seen in each of the three molecules of the asymmetric unit and is also present in the lower resolution structure of ml8bTIM (PDB entry 1DKW). In the wild-type loop-7, the side chain of Val214 is seen to have an anchoring role, and its repositioning in ml8bTIM caused by the new position of the Val233 side chain is not consistent with the catalytic function of loop-7. In two other respects, this new position of the Val233 side chain interferes also with the proper active site geometry. (i) The Val233 side chain overlaps with a conserved water binding site, and therefore no water can be bound at this site. A water molecule is bound at this site in the unliganded as well as the liganded structure of wtTIM (Kursula et al., 2003, 2004). This water (water-1 in Fig. 4) is hydrogen bonded with both loop-7 as well as loop-8, and it therefore appears to be an intrinsic feature of the competent active site. (ii) The Val233 side chain comes close to the position of the phosphate moiety of the substrate and therefore could interfere also directly with substrate binding. These observations suggest that mutating Val233 of ml8bTIM into an alanine would generate an active site with more favorable binding properties. Therefore, this variant (A-TIM) was made and its label figures when necessary. Stereo pairs were created using the convert command of program ImageMagick version 6.6.4 (http://www.imagemagick.org).

Fig. 2. Electron density (Fo-Fc) omit maps. The maps have been calculated after omit refinement, leaving out the highlighted moiety from the model. (A) The A-TIM-CA structure, after omitting the citrate molecule (contoured at 2.5 sigma). (B) The A-TIM-2PG structure, after omitting the 2PG molecule (contoured at 3.0 sigma). (C) The A-TIM-BHAP structure, after omitting BHAP and Glu167 (contoured at 3.0 sigma).
crystallographic and enzymological properties are described in the next sections.

The crystal structure of A-TIM, cocrystallized with citrate
A-TIM has been crystallized with two molecules per asymmetric unit. The crystals are well ordered and the structure has been refined at 1.6 Å resolution. The cores of the two molecules are identical, with an rmsd value of 0.2 Å for the corresponding Ca-atoms of the eight β-strands. In both molecules, loop-6 and loop-7 have adopted the closed conformation and a citrate molecule, present as the buffer ion in the mother liquor, is bound in each active site (Fig. 2). The crystal contacts of this crystal form have caused a large rearrangement in loop-1 and smaller differences in the Ca-traces of loop-2 when comparing molecules A and B. The active site of molecule A is involved only loosely in crystal contacts, whereas this region of molecule B is involved in extensive crystal contacts. The largest difference is seen for residue Pro18 in the middle of loop-1 which has shifted by 8 Å when comparing molecules A and B. The mode of binding of the citrate molecule is well defined by its electron density map (Fig. 2) in both molecules and the same mode of binding is seen for the active sites of molecules A and B. Figure 4 compares the mode of binding of citrate with the mode of binding of the transition state analog 2PG to wild-type TIM. From this superpositioning, it can also be seen that the conformations of loop-6 and loop-7 of A-TIM and wtTIM are indeed very similar. It is also seen that the side chain of Val214 of A-TIM and wtTIM are indeed very similar. It is also seen that the side chain of Val214 of loop-7 is not pushed aside, as observed in ml8bTIM (Fig. 2). Also water-1 is now present, being hydrogen bonded to loop-7 and loop-8. The mode of binding of citrate is very interesting, as it is observed to have the same key interactions with the protein as seen for the transition state analog 2PG: (i) the carboxylate moiety bound in the catalytic site superimposes well on the carboxylate moiety of 2PG and (ii) the anchoring hydrogen bonding interactions of the phosphate oxygen atom of 2PG with loop-6 and loop-7 is also preserved in the citrate mode of binding (Fig. 4). The citrate molecule has a central carbon atom, which has two acetylate groups, one carboxylate group and one hydroxyl group (Fig. 1). One of the acetylate groups corresponds to the acetylate group of the transition state analog 2PG (Fig. 4) and the other acetylate group is involved in anchoring hydrogen bonding interactions to loop-6 and

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**Fig. 3.** The comparison of ml8bTIM and wtTIM. (A) Comparison of the Ca-traces of ml8bTIM (cyan) and wtTIM (magenta, subunit A of 5TIM). The arrow marks the conformational differences of the loop-7 region. The side chains of the active site residues Asn11, Lys13, His95 and Glu167 are also shown. (B) Zoomed in view. The asterisk marks the region of Val214 and Val233.

**Fig. 4.** Comparison of A-TIM-CA and wtTIM. Zoomed in view of the active site region of A-TIM-CA (cyan, complexed with citrate), and wtTIM (magenta, complexed with 2PG). In the structure of the latter complex (1N55), the 2PG molecule is seen in a double conformation. The colored spheres highlight water-1.
loop-7. The hydroxyl group points to bulk solvent and the remaining carboxylate group points into the new binding groove, unique for ml8bTIM and A-TIM and not present in wild-type TIM. Therefore, this mode of binding of citrate is not possible in wtTIM and indeed citrate is not an inhibitor of wtTIM (see below).

The enzymological characterization of A-TIM

A-TIM is a marginally stable protein, as can be deduced from the CD-melting experiments (Fig. 5). The $T_m$ calculated from these melting curves is 44°C, which is lower as found previously for other monomeric TIM’s (49°C) (Schliebs et al., 1996; Thanki et al., 1997). In the presence of 2 mM 2PG, the melting curve shifts to higher temperatures and the $T_m$ value becomes 48°C, indicating that 2PG does bind to A-TIM. The shift of the $T_m$ value of 4°C is in the same range as observed previously for other variants of monomeric TIM (Schliebs et al., 1996; Thanki et al., 1997). For a similar experiment done with ml8bTIM, the melting curves in the absence and presence of 2PG were virtually the same, suggesting no binding of 2PG (Norledge et al., 2001). Control experiments aimed at verifying possible catalytic TIM activity of A-TIM showed no detectable TIM activity for D-GAP as a substrate, when using the linked assay for detection of the product DHAP by the linker enzyme GDH. The binding of citrate to A-TIM, as seen in the crystal structure, is a unique A-TIM property. wtTIM was shown not to be inhibited by citrate at concentrations of up to 5 mM, indicating no affinity of wtTIM for citrate.

Crystallographic binding studies with A-TIM

Three different binding studies with respectively a transition state analog (2PG), a suicide inhibitor (bromohydroxyacetone phosphate, BHAP) and a substrate analog (G3P) were done. In these binding studies, the citrate of the mother liquor was omitted from the soaking solutions and replaced by the respective active site ligand. The binding studies with G3P resulted in the structure of an unliganded, apo A-TIM structure in molecule A, whereas molecule B still had bound citrate.

The mode of binding of the transition state analog, 2PG

Following the protocol described in the methods section a 2.3 Å data set could be collected from a crystal soaked in the mother liquor, supplemented with 10 mM 2PG, but omitting the citrate additive. The data collection and refinement statistics are given in Table II. In each of the two molecules, a molecule of 2PG has replaced the bound citrate (Fig. 2). Figure 6 compares the structure of the A-TIM-2PG complex with the structures of the corresponding complexes of leishmania TIM (Kursula et al., 2003) and the monomeric ml1TIM (Thanki et al., 1997). It is seen that the active site geometry and the mode of binding of 2PG in each of the two monomeric TIM complexes is very similar, but not identical. For example, the molecule is shifted outwards by 0.9 Å when comparing the 2PG mode of binding to A-TIM with its wtTIM and ml1TIM mode of binding. The catalytic glutamate adopts the catalytically competent swung-in conformation (Fig. 6).

Fig. 5. CD-melting curves of A-TIM in the absence of a ligand (O) and in the presence of 2 mM 2PG (triangle).

Fig. 6. Comparison of A-TIM-2PG with wtTIM and ml1TIM. (A) Stereo view of the active site regions of A-TIM-2PG (cyan) and wtTIM, complexed with 2PG (magenta, 1N55). In the structure of the latter complex (1N55), the 2PG molecule is seen in a double conformation. (B) Stereo view of the active sites of A-TIM-2PG and ml1TIM, complexed with 2PG (magenta, 1ML1). The colored spheres mark the positions of water-1.
The new, extended binding pocket of A-TIM, near the phosphate moiety of 2PG, due to the shortening of the phosphate binding loop, is visualized in Fig. 7. The extended groove is lined by residues of loop-7 and loop-8 and extends till the side chains of Phe242 and Ile245, located at the bottom of the groove. Preliminary docking calculations suggest that linear alkyl chain extensions of the 2PG-phosphate moiety with a length of approximately six carbons would fill this binding groove. Such compounds, sulphonate analogs of 2PG and DHAP, are currently being synthesized as possible binders and substrates. The chemical properties of these analogs are such that the elimination side reaction (Richard, 1984), which is an important issue when considering the triosephosphates as substrate, is not relevant. Crystallographic and NMR studies with such substrate analogs will be done to verify the mode of binding of the extended alkyl chains in this new binding pocket and to establish if such compounds are true substrates.

The mode of binding of the suicide inhibitor, BHAP

BHAP is a suicide inhibitor of TIM (Coulson et al., 1970; Mare De la et al., 1972; Norton and Hartman, 1972), like glycidol-phosphate (Rose and O’Connell, 1969; Waley et al., 1970; Schray et al., 1973). BHAP and glycidol-phosphate have been used in the classical studies aimed at identifying the catalytic base responsible for the catalytic function of TIM. From a crystal soaked in the presence of this suicide inhibitor, a data set with a resolution of 2.2 Å could be collected. The refined structure shows that the active site glutamate of molecule B has fully reacted with the suicide inhibitor, whereas in molecule A, possibly a mixture of a derivatized glutamate and a bound citrate, with low occupancy, is present. The omit density of the BHAP-glutamate derivative of molecule B is visualized in Fig. 2, confirming the ester formation between dihydroxyacetone-phosphate and the catalytic glutamate (Mare De la et al., 1972). A structure of this covalent complex has not yet been reported for wild-type TIM. No other glutamates are seen to be covalently modified in this experiment, indicating that the A-TIM active site glutamate is more reactive than any of the other glutamates elsewhere in the structure of A-TIM. The structural comparisons (Fig. 8) show only minor adjustments of the side chain of the catalytic glutamate when comparing the structures of the A-TIM-BHAP and wtTIM-2PG complexes. Interestingly, the BHAP molecule is bound closer to the catalytic site, e.g. its phosphate moiety has moved by ~0.5 Å toward the catalytic site, when compared with the 2PG mode of binding to A-TIM (Figs 6 and 8).

The apo-structure of A-TIM

The soaking protocol in which the mother liquor was supplemented with 10 mM G3P resulted in a crystal structure at 2.0 Å resolution in which the active site of molecule A is unliganded, whereas the active site of molecule B has still a bound citrate molecule. The observation of the empty binding pocket of molecule A indicates that the A-TIM affinity for G3P is much weaker as for 2PG, which agrees with the ~20-fold lower G3P-versus-2PG affinity seen for wtTIM (Lambeir et al., 1987). A comparison of the structures of the unliganded active sites of molecule A and wtTIM shows that loop-6 and loop-7 have adopted the open, unliganded conformation. The tip of loop-6 of molecule A has high B-factors, as usually seen for the open conformation of this loop.

The catalytic and binding properties of the A-TIM active site

The absence of catalytic turnover by A-TIM is consistent with previous observations by Knowles and coworkers (Pompliano et al., 1990) who studied the properties of a chicken TIM variant in which the phosphate binding region of loop-6 was deleted. In this variant, the catalytic activity for both substrates was reduced by approximately a factor 100 000, whereas the binding affinity for both substrates was found to be 10-fold lower. There are no crystal structures available of this loop-6 mutant. Interestingly, for this mutant, it was shown that the methylglyoxal synthase activity is increased. This phosphate elimination reaction is known to be much favored in catalyzes by a non-enzymatic base...
(Richard, 1984) and this increase of methylglyoxal synthase activity was also noted for this chicken TIM variant. In TIM, the phosphate moiety has tight interactions with loop-6, loop-7 and loop-8. It seems likely that the precise mode of binding of the phosphate moiety of the substrate anchored by its interactions (in wild-type TIM) with loop-6, loop-7 and loop-8 is critical for preventing the methylglyoxal synthase reaction and for the efficient catalysis of the isomerase reaction (Pompliano et al., 1990). Indeed, when comparing the precise mode of binding of 2PG to A-TIM and wtTIM, small differences are found in the ligand and protein part; for example, its phosphate moiety is shifted 0.9 Å outwards in the A-TIM complex with respect to the wtTIM and ml1TIM complexes (Fig. 6). It appears that the loop-6 conformation has shifted similarly (Fig. 6). There are also small structural rearrangements for the catalytic side chains, e.g. of Asn11, Lys13 and His95 (Fig. 6), which will affect the precise mode of binding of the substrate. The mode of binding of 2PG to A-TIM is less compact, when compared with wild type. The importance of the compactness of the complexed TIM active site for efficient catalysis has been emphasized recently (Jogl et al., 2003). Interestingly, in the protein engineering efforts of the conversion of a ribose-phosphate binding protein into a TIM, the active site is completely buried in the ribose-phosphate binding protein (Dwyer et al., 2004). In this case, a mutated variant, novoTIM, has been obtained with significant catalytic activity (Dwyer et al., 2004; Allert et al., 2007). The mode of binding of 2PG to A-TIM, as visualized in Fig. 7, is rather solvent exposed, which is different from its mode of binding in wild-type TIM and which is also different from the presumed mode of binding of the substrate in novoTIM.

Summarizing, it is shown that, despite the new extended binding groove, the active site of A-TIM can bind competently transition state analogs and suicide inhibitors of the wild-type TIM. In addition, it is found that the active site can bind a citrate molecule. The binding of this molecule, devoid of a phosphate moiety, is stabilized by interactions with both the new binding groove. Loop-6 and loop-7 adopt the closed conformation in these complexes and the catalytic glutamate is seen in the active ‘swung-in’ conformation. The geometry of the complexed A-TIM active site is less compact and more solvent exposed when compared with the wtTIM complex. The binding and catalytic properties of A-TIM will now be explored further, by doing X-ray and NMR studies of A-TIM with substrate analogs with extended alkyl chains designed to bind in the new binding pocket between loop-7 and loop-8.

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References

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