High resolution crystal structures of triosephosphate isomerase complexed with its suicide inhibitors: The conformational flexibility of the catalytic glutamate in its closed, liganded active site

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Abstract: The key residue of the active site of triosephosphate isomerase (TIM) is the catalytic glutamate, which is proposed to be important (i) as a catalytic base, for initiating the reaction, as well as (ii) for the subsequent proton shuttling steps. The structural properties of this glutamate in the liganded complex have been investigated by studying the high resolution crystal structures of typanosomal TIM, complexed with three suicide inhibitors: (S)-glycidol phosphate ((S)-GOP, at 0.99 Å resolution), (R)-glycidol phosphate, ((R)-GOP, at 1.08 Å resolution), and bromohydroxyacetone phosphate (BHAP, at 1.97 Å resolution). The structures show that in the (S)-GOP active site this catalytic glutamate is in the well characterized, competent conformation. However, an unusual side chain conformation is observed in the (R)-GOP and BHAP complexes. In addition, Glu97, salt bridged to the catalytic lysine in the competent active site, adopts an unusual side chain conformation in these two latter complexes. The higher chemical reactivity of (S)-GOP compared with (R)-GOP, as known from solution studies, can be understood: the structures indicate that in the case of (S)-GOP, Glu167 can attack the terminal carbon of the epoxide in a stereoelectronically favored, nearly linear O—C—O arrangement, but this is not possible for the (R)-GOP isomer. These structures confirm the previously proposed conformational flexibility of the catalytic glutamate in its closed, liganded state. The importance of this conformational flexibility for the proton shuttling steps in the TIM catalytic cycle, which is apparently achieved by a sliding motion of the side chain carboxylate group above the enediolate plane, is also discussed.

Keywords: atomic resolution; TIM; dynamics; reaction mechanism; enolising enzyme

Abbreviations: BHAP, bromohydroxyacetone phosphate; D-GAP, D-glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; IPP, isohydroxamate phosphopropionate; 2PG, 2-phosphoglycolate; PGH, phosphoglycolohydroxamate; (R)-GOP, (R)-glycidol phosphate; (S)-GOP, (S)-glycidol phosphate; TIM, triosephosphate isomerase.

The structures have been deposited at the RCSB with the following access codes (S)-GOP (2Y61), (R)-GOP (2Y62), and BHAP (2Y63).

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Figure 1. (A) The TIM reaction. Glu167 is the catalytic base which generates the initial enediolate intermediate from the substrate, DHAP. Several additional proton shuttling steps complete the reaction cycle to the product, D-GAP.^{1,2} (B) The covalent structures and nomenclatures of the (R)-GOP, (S)-GOP, and BHAP suicide inhibitors. The systematic name of GOP is glycidyl phosphate. The (S)-GOP enantiomer corresponds to D-GOP and has the same chirality as the substrate D-GAP. The (R)-GOP enantiomer corresponds to L-GOP. The covalent structures of the transition state analog 2PG, and the reaction intermediate analogs PGH and IPP are also shown. PGH is a mimic of the ketoform of the substrate³ and IPP is a mimic of the aldehyde form of the substrate.⁴ (C) The covalent modification reaction of (S)-GOP. (D) The covalent modification reaction of BHAP. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Introduction

Triosephosphate isomerase (TIM) (EC 5.3.1.1)^{1,2} is a homodimeric enzyme, interconverting an α -hydroxyketone (dihydroxyacetone phosphate, DHAP) and an α -hydroxyaldehyde (D-glyceraldehyde-3-phosphate, D-GAP; Fig. 1). The enzyme is highly specific for D-GAP and has much lower affinity and catalytic efficiency for L-GAP.⁵ TIM catalyzes an essential step in the glycolytic pathway. A key discovery in the early studies of its reaction mechanism has been the finding that a glutamate, Glu167 in trypanosomal TIM, is a catalytic residue.^{6–8} This discovery

was made using the suicide inhibitors glycidol phosphate (GOP)⁹ and bromohydroxyacetone phosphate (BHAP)¹⁰ (Fig. 1). These suicide inhibitors inactivate TIM with about the same second-order rate constants,^{10,11} being $\sim 10^4 \text{ M}^{-1} \text{ s}^{-1}$. The reaction of the epoxide (R)/(S)-GOP results in a glycerol phosphate ester of Glu167, whereas the reaction with BHAP results in the formation of a DHAP ester of Glu167 (Fig. 1). In these complexes, the total number of ligand atoms in the active site is one oxygen atom less (the O1 oxygen atom is missing in the suicide inhibitors) as in the enzyme–substrate complex with

Figure 2. The competent active site at the dimer interface of the TIM-PGH complex (2VXN). The dotted lines mark the hydrogen bonding network between O2(PGH), NZ(Lys13), Glu97, Thr75*, and Asn11, including the water mediated hydrogen bond between NZ(Lys13) and the phosphate moiety. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DHAP¹² (Fig. 1) and enzyme-reaction intermediate complexes with phosphoglycolohydroxamate, PGH¹³ and isohydroxamate phosphopropionate, IPP⁴ (Fig. 1). The latter two complexes mimic the structures of the enzyme-substrate and enzyme-product complexes, respectively.⁴ Further studies revealed that (*S*)-GOP reacts 10 times faster than (*R*)-GOP.¹⁴ The halogen-hydroxyacetone sulfates and phosphates (like BHAP) have also been used to measure the pK_a of this catalytic glutamate,¹⁵ being 3.9 for the unliganded enzyme.

Figure 2 displays the active site of TIM, which is built around the four catalytic residues Asn11, Lys13, His95, and Glu167, at the dimer interface. Thr75* of the adjacent subunit is part of the active site hydrogen bonding network, below the enediolate plane. This hydrogen bonding network also includes the side chains of Glu97, being hydrogen bonded to Lys13 and Thr75*. Thr75* is subsequently hydrogen bonded to ND2(Asn11) (Fig. 2). A key feature of the active site is also the presence of flexible Loop-6 (residues 168–178), which in a concerted fashion, together with Loop-7 (residues 210–213), closes on substrate binding.¹⁶ The catalytic glutamate, Glu167, is at the beginning of Loop-6. The side chain of Glu167 is moved into its catalytically competent conformation concomitantly on the closure of Loop-6. Glu167 is the catalytic base of this enolizing enzyme, which abstracts the proton from the substrate, gen-

erating the enediolate intermediate (Fig. 1). This proton abstraction initiates the catalytic cycle. The negative charge of the enediolate intermediate (Fig. 1) is stabilized by favorable hydrogen bonds with NZ(Lys13) and NE2(His95). The Glu167 side chain is located above the enediolate plane (Fig. 2). In its closed conformation, the side chain of Glu167 has additional conformational flexibility, which is proposed to be important for the proton shuttling of the catalytic cycle.^{2,17} In this conversion, protons are transferred from the C1/O1 moiety of the substrate to the C2/O2 moiety of the product (Fig. 1). Two proton shuttling mechanisms have been proposed.^{1,2} In the criss-cross mechanism,^{18,19} all proton transfer steps are done by the catalytic glutamate. In the classical mechanism,^{20,21} the side chains of His95 and Glu167 are both involved in the proton shuttling. The side chain of His95 is anchored by a hydrogen bond between the unprotonated ND1(His95)¹⁷ and N(Glu97) (Fig. 2). These subsequent proton shuttling steps complete the conversion of the α -hydroxyketone into the α -hydroxyaldehyde, after the initial proton abstraction step (Fig. 1).

In the studies reported here, we describe the crystal structures of the complexes of TIM with (*S*)-GOP, (*R*)-GOP, and BHAP. The crystal structures of the (*S*)-GOP and (*R*)-GOP TIM complexes have been refined at atomic resolution. These structures provide a rationale for the higher reactivity of (*S*)-GOP

Table I. *Data Collection Statistics*

Ligand	(S)-GOP	(R)-GOP	BHAP
Beamline	X12	X12	Home source
Wavelength (Å)	0.8997	0.8997	1.5418
Temperature (K)	100	100	100
Space group	<i>C2</i>	<i>C2</i>	<i>C2</i>
V_m (Å ³ Da ⁻¹)	2.3	2.3	2.3
No. of subunits/asu	1	1	1
Unit cell			
a, b, c (Å)	97.14, 52.47, 58.09	97.76, 52.46, 58.25	99.88, 50.66, 58.83
β (°)	117.85	118.08	118.14
Resolution range (Å)	10.0–0.99 (1.02–0.99)	10.0–1.08 (1.10–1.08)	99.0–1.97 (2.04–1.97)
R_{merge} (%)	6.7 (44.2)	9.2 (42.0)	4.4 (5.4)
R_{meas} (%)	4.7 (44.3)	7.1 (45.0)	—
I/σ	21.4 (3.8)	16.2 (3.6)	29.1 (20.7)
Completeness (%)	97.7 (95.3)	99.0 (97.7)	98.8 (97.0)
Redundancy	5.5 (4.0)	6.1 (4.0)	3.9 (3.3)
No. of unique reflections	140,160 (11,620)	110,340 (5806)	18,363 (1793)
Wilson B -factor (Å ²)	10.5	10.7	21.0
Processing software	XDS	XDS	Denzo/Scalepack

as compared with (*R*)-GOP. The three structures confirm the conformational flexibility of the Glu167 side chain in its closed, liganded conformation. It is discussed how the conformational flexibility of the Glu167 side chain can facilitate its proton shuttling steps in the catalytic cycle of TIM.

Results and Discussion

The structures of the (*R*)-GOP and (*S*)-GOP complexes have been refined at atomic resolution, at 0.99 and 1.08 Å, respectively, whereas the crystal structure of the BHAP-complex has been refined at 1.97 Å resolution. For each structure, the data

Table II. *Refinement Statistics*

Ligand	(S)-GOP	(R)-GOP	BHAP
Resolution range (Å)	10.0–0.99	10.0–1.08	51.98–1.97
Protein atoms	2013	2050	1941
Ligand atoms	18 ^{a*}	18 ^{a*}	9
Other solute atoms	12	12	0
Water molecules	417	395	429
R_{work} (For $F_o > 4\sigma(F_o)$; %)	9.5	9.9	—
R_{free} (For $F_o > 4\sigma(F_o)$; %)	11.4	12.2	—
R_{work} (all data; %)	10.6	10.9	13.5
R_{free} (all data; %)	12.5	13.3	17.0
Geometry			
RMSD bond length (DFIX; Å)	0.017 (0.020)	0.015 (0.020)	0.008
RMSD bond angle (DANG; Å)	0.034 (0.040)	0.032 (0.040)	—
RMSD bond angle (°)	—	—	1.1
Non-zero chiral volume (Å ³)	0.102 (0.100)	0.092 (0.100)	
Zero chiral volume (Å ³)	0.105 (0.100)	0.091 (0.100)	
DELU (Å ²)	0.006 (0.010)	0.005 (0.010)	
SIMU (Å ²)	0.038 (0.138)	0.037 (0.138)	
ISOR (Å ²)	0.094 (0.100)	0.087 (0.100)	
Average B -factor (Å ²)			
Protein main chain	9.8	8.2	6.0
Protein side chain	13.1	11.5	7.3
Ligand	9.9	6.7	5.4
Other solute molecules	15.2	11.6	—
Water molecules	28.4	25.5	21.9
Ramachandran plot (%)			
Most favored regions	94.1	93.7	93.7
Allowed regions	5.9	6.3	6.3
Outliers	0	0	0
PDB entry	2Y61	2Y62	2Y63

^a Both enantiomeric forms of the ligand have been built. In the (*S*)-GOP complex the occupancy of (*S*)-GOP is 73% (the (*R*)-GOP occupancy is 27%), whereas in the (*R*)-GOP complex the occupancy of (*R*)-GOP is 76% (the (*S*)-GOP occupancy is 24%).

Figure 3. The omit map of (S)-GOP, calculated after omit refinement at 0.99 Å resolution. These omit calculations were done with a model obtained from the final (S)-GOP structure by deleting the inhibitor atoms as well as the Glu167 side chain atoms. The atoms of both the major form ((S)-GOP) and the minor form ((R)-GOP) of GOP as well as the Glu167 atoms of the final (S)-GOP structure are shown. The map is contoured at 2.8σ . [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

collection and refinement statistics are reported in Tables I and II, and for each structure, the mode of binding of these suicide inhibitors is clearly defined by the electron density maps. In the complexes with (R)-GOP and (S)-GOP, residual electron density attributable to the Glu167 esters of the other enantiomeric GOP isomer can be identified (Table II). The presence of both forms in each active site shows that a mixture of bound (S)-GOP and (R)-GOP has been captured in these experiments. Figure 3 shows the omit density after omit refinement of the (S)-GOP complex calculated by omitting the atoms of (S)-GOP and side chain of Glu167. In the structure of the (S)-GOP complex, the chiral hydroxyl atom (O2) points downward (Fig. 4), toward the Asn11 side chain. In the (R)-GOP structure, this hydroxyl moiety points toward the His95 side chain. In each of the structures, this oxygen is hydrogen bonded to NE2(His95), like in the competent active site. In the (R)-GOP structure the conformations of the side chains of Glu97 and Glu167 have changed, but not in the (S)-GOP structure. In every respect, the side chain dihedrals of Glu97 and Glu167 of the (S)-GOP

active site are the same as seen in previous TIM-complexes (Table III). This geometry will be referred to as the geometry of the competent active site (Fig. 4).

The structural changes of Glu97 and Glu167 in the (R)-GOP complex (Fig. 5) concern mostly changes of the side chain dihedrals of these two residues (Table III), as compared with the competent active site. The dihedrals of Glu97 and Glu167 in the (R)-GOP complex are associated with less favorable, more strained conformations.^{23,22} Figure 6 shows that in the (R)-GOP structure, the carboxylic end of the Glu167 side chain is rotated toward a similar, but not identical, position as seen in the complex of TIM with the reaction intermediate analog IPP, whereas in the (S)-GOP conformation (Fig. 5), the Glu167 side chain conformation corresponds to the conformation seen in the TIM-PGH complex. In the latter complex (mimicking the enzyme substrate, DHAP, complex), the side chain is positioned near Cys126/Ala165, whereas in the IPP complex (mimicking the enzyme product, D-GAP, complex) it is more close to Ile172 (Fig. 4).

Figure 4. The comparison of the active site geometry of unliganded TIM (purple, 5TIM) and the active sites complexed with IPP (cyan, 1IF2), 2PG (magenta, 1N55), DHAP (green, 1NEY) and PGH (yellow, 2VXN). The comparison highlights the conformational flexibility of Glu167 in the competent active site.

Ile172 is a highly conserved residue; it is interesting to note that the I172V mutation in human TIM is associated with a TIM deficiency disease.²

The side chain of Glu97 is in each of the currently characterized competent complexes always involved in a hydrogen bonded network with Lys13 and Thr75*, below the enediolate plane (Fig. 2). OE1(Glu97) in its (*R*)-GOP conformation, however, is hydrogen bonded with the main chain N(Glu97) atom. The main chain of Glu97 in the suicide inhibitor complex is somewhat displaced, and the orientation of the His95 side chain is also slightly changed in the (*R*)-GOP complex. Possibly, the repositioning of the Glu167 side chain causes the rearrangement of the His95 side chain, which then subsequently is correlated with the repositioning of the Glu97 side chain and main chain. The OE1(Glu97)-N(Glu97) hydrogen bonding interaction is uniquely different from the competent complex, where always a good hydrogen bond between ND1(His95) and N(Glu97) is observed. Interestingly, in its new conformation, the OE1(Glu97) oxygen is also hydrogen bonded to ND1(His95). The (OE1)Glu97-ND1(His95) distance is 2.7 Å, indicating that ND1(His95) has become protonated and therefore, suggesting that His95 is posi-

tively charged in this complex. The OE2(Glu97) remains hydrogen bonded to OG1(Thr75*) in both conformations. The direct Glu97-Lys13 salt bridge of the competent active site is replaced by a water mediated Glu97-Lys13 salt bridge in the (*R*)-GOP suicide complex (Fig. 5). There are no other structural changes in the active site of the (*R*)-GOP complex.

The structure of the BHAP complex has been refined at 1.97 Å resolution (Table II). The mode of binding of BHAP is clearly defined by its electron density map. In the BHAP complex, similar conformational changes are seen for the side chains of Glu97 and Glu167 as in the (*R*)-GOP complex (Table III and Fig 5). The orientation of the His95 side chain is also slightly changed in the BHAP complex, like in the (*R*)-GOP complex. Like in the (*R*)-GOP complex, OE1(Glu97) is hydrogen bonded to ND1(His95) and N(Glu97). In the BHAP complex, the keto oxygen (O2) is hydrogen bonded to (NE2)His95 (Fig. 5), like the chiral hydroxyl oxygen (O2) of (*R*)-GOP.

The changes of the side chain conformations of Glu97 and Glu167 in the (*R*)-GOP complex, as compared with wild type correlate with the 10-fold lower

Table III. The Side Chain Conformations of Glu97 and Glu167

PDB entry	5TIM-A	2VXN	1NEY	1N55	1IF2	2Y61	2Y62	2Y63
Ligand	unliganded	PGH	DHAP	2PG	IPP	(<i>S</i>)-GOP	(<i>R</i>)-GOP	BHAP
Glu167								
χ_1 (°)	-58	-45	-47	-53	-57	-50	-180	-180
χ_2 (°)	-175	-162	-170	-171	-158	-169	155	158
Glu97								
χ_1 (°)	-45	-68	-66	-69	-74	-66	72	80
χ_2 (°)	-162	150	153	152	146	151	-68	-72

5TIM-A is the unliganded open conformation (subunit A of 5TIM). 2VXN is the liganded, closed conformation (the ligand is the reaction intermediate analog PGH). 1NEY is the liganded, closed conformation, in the presence of the substrate DHAP. 1N55 is the liganded, closed conformation in the presence of the transition state analog 2PG. 1IF2 is the liganded, closed conformation (the ligand is the reaction intermediate analog IPP). The side chains of Glu97 and Glu167 adopt the preferred, unstrained, conformations²² only in the unliganded, PGH, DHAP, 2PG, IPP, and (*S*)-GOP structures, visualizing the competent active site (Fig. 2).

Figure 5. The structures of the (*S*)-GOP complex (blue), (*R*)-GOP complex (magenta), and the BHAP-complex (green), compared with the PGH complex (yellow, 2VXN). Thr75*, Glu97, Asn11, Lys13, His95, and Glu167 are also shown. A water molecule (O, orange) replaces the Glu97 side chain in the (*R*)-GOP and BHAP structures.

reactivity of (*R*)-GOP over the (*S*)-GOP reactivity. It is possible that the activation energy, associated with the side chain rotations to these new conformations (Table III), is causing the lower reactivity of the (*R*)-GOP reactant. An alternative possibility, also consistent with the structural evidence, is that the

activation energy of the chemical reaction of making the covalent bond with the (*R*)-GOP reactant is higher than for the (*S*)-GOP reactant for stereoelectronic reasons. The ring opening of the epoxide is subject to general acid catalysis.^{24,25} The complexed active site is very tight,^{12,17} suggesting that there

Figure 6. The structure of (*R*)-GOP (magenta) compared with the IPP complex (cyan, 1IF2) and the PGH complex (yellow, 2VXN), visualizing the hydrophobic cage of the Glu167 side chain, shaped by Cys126, Ala165, Ile172, Gly211, and Leu232. The Glu167 side chain is near Cys126, Ala165 in the PGH complex, mimicking the enzyme-DHAP complex, and near Gly211 (Loop-7), Ile172 (Loop-6) in the IPP complex, mimicking the enzyme-D-GAP complex.⁴

will not be a major conformational rearrangement of the suicide inhibitor on ring opening. Therefore, the GOP epoxide oxygen in each of the GOP–TIM complexes will be positioned near NZ(Lys13), suggesting that NZ(Lys13) is the general acid for the ring opening (Fig. 1). In this scenario, the epoxide ring in the (*R*) and (*S*) isomers could be opened by the attacking nucleophile, the OE2 of Glu167. The stereoelectronically optimal angle of attack for a S_N2-type epoxide ring opening involves about 150–160° O–C–O angle among the nucleophilic oxygen (in this case the OE2 of Glu167), the terminal carbon of the epoxide, and the epoxide oxygen.^{26–28} The structures of the complexes of the covalently attached (*S*)-GOP and (*R*)-GOP suicide inhibitors indicate that in the (*S*)-GOP–TIM structure this OE2(Glu167)–C(terminal carbon of GOP)–O(epoxide) angle is 151°, indicating an almost ideal geometry, whereas in the (*R*)-GOP–TIM complex, this O–C–O angle is 99° (Figs. 3 and 5). The corresponding dihedral OE2(Glu97)–C3–C2–O2(GOP) angles are –172° for the (*S*)-GOP complex and –70° for the (*R*)-GOP complex. Therefore, the differences in the experimentally observed faster rate of ring opening for the (*S*)-isomer of GOP correlates nicely with the stereoelectronically more favorable geometry of the ring opening in the (*S*)-GOP complex.

It is known that in the liganded TIM complexes the side chain of the catalytic glutamate is pointing into a hydrophobic cage,²⁹ above the enediolate plane, defined by the side chains of Cys126/Ala165 and Ile172 of Loop-6 (Fig. 4). In this cage, the side chain is poorly hydrogen bonded,² however, tightly interacting with the reactive end of the substrate.¹² It adopts this buried conformation on ligand binding, when Loop-6 and Loop-7 are in the closed conformation. In this geometry, its pK_a is raised,²⁹ facilitating its role as the catalytic base (Fig. 1). The structural comparison shown in Figure 6 highlights that the conformational flexibility of the catalytic glutamate in the R-GOP complex is achieved by side chain rotations, whereas in the IPP and PGH complexes, it is achieved by a sliding motion of the Glu167 side chain above the enediolate plane. This sliding motion is also shown in Figure 4, providing a more detailed structural comparison, including the structures with the transition state analog 2PG³⁰ and the structure with the substrate DHAP.¹² The side chain dihedrals of the Glu167 side chain remain the same in each of these liganded structures (Table III). It is interesting to note that these side chain dihedrals are also the same as observed in the unliganded, open conformation (Table III). Apparently, the movement of the Glu167 side chain from open, unliganded to closed, liganded (Table III) as well as within the closed, liganded structure (Table III), does not involve side chain rotations (Fig. 4 and Table III), but is achieved by small structural adjust-

ments in the Glu167 main chain conformation. In the liganded structures, these structural adjustments cause a sliding motion of the Glu167 carboxylate moiety above the enediolate plane, ranging from the geometry seen in the TIM–PGH complex to the geometry seen in the TIM–IPP complex. In the former complex, the Glu167 side chain is near the C1/O1 moiety of the substrate, whereas in the latter complex, it is near the C2/O2 moiety of the product (Fig. 1). Further studies, by for example NMR^{31–33} and computational approaches,³⁴ are required to provide more insight into the dynamics of the TIM active site.

The new structures of the (*R*)-GOP, (*S*)-GOP, and BHAP complexes of TIM confirm the conformational flexibility of the side chain of the catalytic glutamate in the enzyme ligand complex. It appears that in the enzyme substrate complex of TIM a sliding motion of this side chain above the enediolate plane of the intermediate allows for the proton shuttling by this active site residue in the catalytic cycle of TIM.

Methods

Chemicals

BHAP was a gift from Augustijns (University of Antwerp, Belgium), it was synthesized as described previously.¹⁶ 2PG³⁰ and PGH¹⁷ were purchased from ProSyntest (Estonia). (*R*)-GOP and (*S*)-GOP were synthesized according to published procedures.^{35,36} The purity was verified by ¹H NMR (400 MHz).

Crystallization

The crystallographic binding studies as reported here have been done with a point mutation variant of leishmanial TIM. The point mutation is E65Q. The sequence numbering follows the numbering of leishmanial TIM, being the same as the well studied and closely related trypanosomal TIM. In this numbering system, the catalytic glutamate is Glu167, which corresponds to Glu165 in yeast and chicken TIM.¹ This E65Q-variant is much more stable than wild-type leishmanial TIM, but equally active.³⁷ The structures of the high quality crystals of this leishmanial TIM cocrystallized with the transition state analogs 2PG³⁰ and PGH¹⁷ have been characterized at atomic resolutions, respectively, 0.83 and 0.82 Å. The enzyme was purified and crystallized as described before.³⁷ Briefly, crystals of complexes with the transition state analog 2PG³⁰ or the reaction intermediate analog PGH¹⁷ were obtained in a hanging drop setup at room temperature with 4 + 4 μL drops, with 21% PEG6000 as the well solution in 0.1 M acetate buffer at a pH of 4.5–5.5 and 1 mM dithiothreitol (reduced) (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM NaN₃, at room temperature. A protein solution of 11 mg/mL in 20 mM Tris/HCl, pH 7.5, 25 mM NaCl, 1 mM DTT,

1 mM EDTA, and 1 mM NaN₃, containing also 10 mM of the ligand (2PG or PGH), was used.

Soaking with (S)-GOP and (R)-GOP

The crystallization ligand (2PG or PGH) was replaced by (S)-/(R)-GOP by soaking at room temperature. In case of the (S)-GOP experiment, the original crystal was grown in the presence of PGH, whereas the (R)-GOP soaking experiment was done with a crystal grown in the presence of 2PG. The crystals were soaked in a drop of the cryosolution (23% glycerol in the crystallization solution, adjusted to 23%PEG6000, pH 5.5) containing also (S)/(R)-GOP (but without the crystallization ligand). Initially soaking experiments with mother liquor, in which (S)/(R)-GOP was dissolved up to 10 mM failed to flush out the cocrystallization ligand. Therefore, (S)/(R)-GOP were introduced into the drop by adding a small amount of powder of these compounds, such that a saturated solution was made. The crystal was incubated for ~30 min in these drops before freezing.

Soaking with BHAP

The crystallization ligand 2PG was replaced by BHAP in a 5-min soak at room temperature in a drop of the cryosolution (23% glycerol in the crystallization solution, adjusted to 23%PEG6000, pH4.5), containing also 10 mM BHAP (but without the crystallization ligand).

Data collection and structure determination

The data of the (S)-GOP TIM and (R)-GOP TIM crystals were collected at the EMBL/DESY beam line X12 in Hamburg, Germany at 100 K, using a 225mm-MARCCD detector. For both crystals, two data sets were collected, one at high resolution (cutoff at 0.95 Å) and one at low resolution (cutoff at 1.36 Å). The data were processed and scaled by XDS³⁸ with the XDSi interface.³⁹ The BHAP dataset was collected with the in-house rotating anode, equipped with a MAR345 image plate detector (cutoff at 1.97 Å). These images were processed with DENZO and SCALEPACK.⁴⁰ The data collection statistics of the three datasets are provided in Table I. 5% of the observed data was used for the free R calculations. The crystals belong to the space group C2 with one subunit per asymmetric unit. The structures were determined using the MOLREP⁴¹ molecular replacement program of the CCP4 package.⁴² The TIM-2PG structure (PDB entry 1N55) was used as the search model after removing water molecules, ligands and alternate conformations.

Structure refinement of the (S)-GOP and (R)-GOP structures

The (R)-/(S)-GOP-TIM structures were initially refined by REFMAC5.⁴³ Further refinement was carried out by SHELX,⁴⁴ initially using overall aniso-

tropic scaling parameters. Model building in the electron density maps was carried out using the program COOT.⁴⁵ Double conformations, active site ligand and water molecules were added as the refinement progressed. The geometry of the covalent link between the suicide inhibitor and the catalytic glutamate was restrained by angle and distance restraints. In both the (R)- and (S)-GOP structures, both chiral forms were built in the active site with ~75% and 25% occupancy for the major and minor forms, that is, in the structure of the (S)-GOP-TIM complex the (S)-GOP occupancy refined to 73% and the (R)-GOP occupancy to 27%. Toward the end of the refinement, riding hydrogen atoms were introduced following the default instructions generated by SHELXPRO,⁴⁴ and also individual anisotropic *B*-factors were refined for all atoms. For water molecules, the anisotropic *B*-factors were restrained using ISOR restraints of SHELX. The refinement statistics are summarized in Table II.

Structure refinement of the BHAP structure

The BHAP structure was refined with REFMAC5. The geometry of the covalent link between the suicide inhibitor and the catalytic glutamate was restrained by angle and distance restraints, as described.⁴³ The refinement statistics are listed in Table II.

Structure analysis

The program COOT⁴⁵ was used for the analysis of the structures. The program BAVEAGE⁴² was used to calculate the average *B*-factors and PROCHECK⁴⁶ and MOLPROBITY⁴⁷ were used to analyze the Ramachandran plots. The structures of unliganded TIM (5TIM, subunit A, 1.83 Å resolution)⁴⁸ and TIM with ligands, in particular 2PG (transition state analog 1N55, 0.83 Å resolution),³⁰ DHAP (substrate, 1NEY, 1.2 Å resolution),¹² PGH (reaction intermediate analog, 2VXN, 0.82 Å resolution)¹⁷ and IPP (reaction intermediate analog, 1IF2, 2.0 Å resolution)⁴ were used for comparison with the current structures. The structures were superposed using the SSM superpose option⁴⁹ of the COOT program. Figure 1 is drawn with the Chemdraw software and Figures 2–6 are generated using the program PyMol (<http://www.pymol.org/>).

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