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Magnitude and Origin of the Enhanced Basicity of the Catalytic Glutamate of Triosephosphate Isomerase

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Abstract

Glu-167 of triosephosphate isomerase from *Trypanosoma brucei brucei* (*Tbb*TIM) acts as the base to deprotonate substrate to form an enediolate phosphate trianion intermediate. We report that there is a large ~6 pK unit increase in the basicity of the carboxylate side chain of Glu-167 upon binding of the inhibitor phosphoglycolate trianion (**I**$^{3-}$), an analog of the enediolate phosphate intermediate, from pK_{EH} ≈ 4 for the protonated free enzyme **EH** to pK_{EHI} ≈ 10 for the protonated enzyme-inhibitor complex **EH•I**$^{3-}$. We propose that there is a similar increase in the basicity of this side chain when the physiological substrates are deprotonated by *Tbb*TIM to form an enediolate phosphate trianion intermediate and that it makes an important contribution to the enzymatic rate acceleration. The affinity of wildtype *Tbb*TIM for **I**$^{3-}$ increases 20,000-fold upon decreasing the pH from 9.3 to 4.9, because *Tbb*TIM exists mainly in the basic form **E** over this pH range, while the inhibitor binds specifically to the rare protonated enzyme **EH**. This reflects the large increase in the basicity of the carboxylate side chain of Glu-167 upon binding of **I**$^{3-}$ to **EH** to give **EH•I**$^{3-}$. The I172A mutation at *Tbb*TIM results in an ~100-fold decrease in the affinity of *Tbb*TIM for **I**$^{3-}$ at pH < 6, and an ~2 pK unit decrease in the basicity of the carboxylate side chain of Glu-167 at the **EH•I**$^{3-}$ complex, to pK_{EHI} = 7.7. Therefore the hydrophobic side chain of Ile-172 plays a critical role in effecting the large increase in the basicity of the catalytic base upon the binding of substrate and/or inhibitors.

We report that the binding of phosphoglycolate trianion to triosephosphate isomerase from *Trypanosoma brucei brucei* (*Tbb*TIM) results in a large > 6 unit increase in the pK_a of the carboxylic acid side chain of the catalytic base Glu-167 and that the hydrophobic side chain of Ile-172 plays a critical role in effecting this increase in basicity. Brønsted acid-base catalysis by amino acid side chains makes an important contribution to enzymatic rate accelerations. This contribution will be enhanced by interactions between the catalytic side chain and bound substrate, or the interactions of catalytic side chains with one another, that lead to an increase in the thermodynamic driving force for the proton transfer, compared to proton transfer at the free side chain in water.[1–3] However, it is exceedingly difficult to design experiments that provide comparisons between the pK_a of an amino side chain during turnover and the corresponding pK_a in water.

Triosephosphate isomerase (TIM) catalyzes the reversible stereospecific 1,2-hydrogen shift at dihydroxyacetone phosphate (DHAP) to give (*R*)-glyceraldehyde 3-phosphate (GAP) by a

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Supporting Information. Experimental procedures for determination of the kinetic parameters for isomerization of GAP and for competitive inhibition by phosphoglycolate. Values of k_{cat} and k_{cat}/K_m for isomerization of GAP, and $(K_i)_{obs}$ for inhibition by phosphoglycolate, for wildtype and I172A mutant *Tbb*TIM (Tables S1 and S2). Dependence on pH of $(K_i)_{obs}$ for wildtype and I172A mutant *Tbb*TIM (Figure S1). Michaelis-Menten plots of initial velocities for isomerization of GAP by wildtype and I172A mutant *Tbb*TIM in the absence and presence of phosphoglycolate (Figures S2 and S3). This material is available free of charge via the Internet at http://pubs.acs.org.

single base (Glu-165 or Glu-167) proton transfer mechanism through enzyme-bound cis-enediolate reaction intermediates (Scheme 1).[4–8] X-ray crystal structures of unliganded *Tbb*TIM reveal the presence of 6 water molecules within 5 Å of the side chain of the catalytic base Glu-167.[9] Closure of the flexible loop 6 of TIM over the bound substrate DHAP[10,11] and transition state/intermediate analogs[12–15] sequesters these ligands from interaction with bulk solvent and results in the displacement of several water molecules from the active site. For example, only two water molecules lie within 5 Å of the side chain of Glu-167 at the complex between TIM from *Leishmania mexicana* and phosphoglycolohydroxamate.[15] An unperturbed pK_a of 3.9 has been determined for the corresponding well-solvated carboxylate side chain of the catalytic base Glu-165 at yeast TIM,[16] and there is a modest 1 – 2 unit increase in the pK_a of this carboxylate group at the Michaelis complex with substrate.[17–19] We have proposed that ligand-driven desolvation of the active site of TIM results in a large increase in the basicity of the catalytic base.[5,20] We present here the results of studies that provide support for this proposal.

Phosphoglycolate (PGA, Chart 1) is a high-affinity competitive inhibitor of TIM and its specificity in binding to TIM is proposed to be similar to that of the enzymatic transition state.[21,22] The pH profiles for competitive inhibition of yeast TIM by PGA,[16] along with the results of NMR studies of the protonation state of the TIM•PGA complex,[23,24] show the following: (1) PGA binds to TIM (**E**) as its trianion ($\mathbf{I^{3-}}$), and (2) that the binding of $\mathbf{I^{3-}}$ is accompanied by the *uptake* of a proton by TIM to give the $\mathbf{EH{\bullet}I^{3-}}$ complex. X-ray crystal structures of TIM complexed with PGA (Figure 1) are consistent with the presence of a hydrogen bond between the anionic carboxylate group of PGA and the protonated side chain of Glu-165 at yeast TIM,[14] or of Glu-167 at *Tbb*TIM,[12] providing strong evidence that the binding of $\mathbf{I^{3-}}$ to **E** is accompanied by protonation of the side chain of the catalytic base. Presumably the combination of the relief of destabilizing electrostatic interactions between the neighboring carboxylate anions of TIM B and $\mathbf{I^{3-}}$, and the formation of an enzyme-inhibitor hydrogen bond, are responsible for the large driving force for protonation of the side chain of the catalytic glutamate at the $\mathbf{E{\bullet}I^{3-}}$ complex to give $\mathbf{EH{\bullet}I^{3-}}$.[25]

Wildtype *Tbb*TIM and the I172A and L232A mutants were prepared as described in our earlier work.[26–28] Initial velocities, v_i (M s^{-1}), for the enzyme-catalyzed isomerization of GAP in the absence and presence of various concentrations of PGA at pH 4.9 – 9.3, 25 °C and I = 0.1 (NaCl) were determined as described in the Supporting Information. The data obtained at each pH were globally fit to eq 1 to give the values of k_{cat}, K_m and $(K_i)_{obs}$ that are reported in Tables S1 and S2 of the Supporting Information, where $(K_i)_{obs}$ is the *observed* inhibition constant determined for *total* PGA ($\mathbf{I^{3-}} + \mathbf{HI^{2-}}$) at the pH of interest. The values of k_{cat} for both wildtype and I172A mutant *Tbb*TIM are pH-independent between pH 5.7 and 9.3 (Table S1). The pH-rate profiles for k_{cat}/K_m for both wildtype and I172A mutant *Tbb*TIM (not shown) exhibit a downward break at pH 6.1, corresponding to the second ionization of the phosphoryl group of the substrate.[17–19] It has been shown that TIM binds and turns over the phosphoryl dianion form of the substrate.[19]

$$\frac{v_i}{[E]} = \frac{k_{cat}[GAP]}{K_m\left(1+\frac{[PGA]}{(K_i)_{obs}}\right)+[GAP]} \quad (1)$$

The values of $(K_i)_{obs}$ determined here for competitive inhibition of wildtype *Tbb*TIM by *total* PGA at I = 0.1 (NaCl) are similar to those reported previously for yeast TIM over a more narrow range of pH at I = 0.05 (KCl), when the difference in the ionic strength is taken into account.[16] The values of $(K_i)_{obs}$ for inhibition of both wildtype and I172A mutant *Tbb*TIM approach a constant value at low pH, where the inhibitor exists mainly as its dianion $\mathbf{HI^{2-}}$ (Table S2). The downward break in the plots of $p(K_i)_{obs}$ against pH (Figure S1

of the Supporting Information) corresponds to pK_a = 6.3 for ionization of $\mathbf{HI^{2-}}$ to form $\mathbf{I^{3-}}$ at I = 0.1 (Chart 1), which is similar to the pK_a of 6.5 at I = 0.05 reported previously.[16] As discussed above, PGA binds as the trianion $\mathbf{I^{3-}}$ to the *protonated* enzyme **EH** resulting in formation of the $\mathbf{EH{\bullet}I^{3-}}$ complex (Scheme 2).[23,24] Therefore, *true* values of K_i for inhibition of the wildtype and mutant *Tbb*TIMs by the PGA trianion $\mathbf{I^{3-}}$ at each pH (Table S2) were calculated from the *observed* values using eq 2, with pK_a = 6.3 for ionization of $\mathbf{HI^{2-}}$ to give $\mathbf{I^{3-}}$.

Figure 2 shows the pH profiles for competitive inhibition of wildtype and I172A mutant *Tbb*TIM by $\mathbf{I^{3-}}$, along with the value of $K_i = 1.2 \times 10^{-5}$ M determined for inhibition of the L232A mutant at pH 8.3. The solid line through the data for the wildtype enzyme, which has a slope of −1.0, was obtained from the fit of the data to eq 3, derived for Scheme 2, with $K_{EH} >> [H^+]$ and $K_{EHI} << [H^+]$. There is a 20,000 fold decrease in K_i on decreasing the pH from 9.3 to 4.9 and the absence of breaks in the pH profile shows that unliganded wildtype *Tbb*TIM exists mainly as **E** at pH 4.9 (pK_{EH} < 4.9), while the **E**•PGA complex exists mainly as $\mathbf{EH{\bullet}I^{3-}}$ at pH 9.3 (pK_{EHI} > 9.3). The data for the I172A mutant were fit to eq 3, with $K_{EH} >> [H^+]$, to give pK_{EHI} = 7.7 for ionization of the $\mathbf{EH{\bullet}I^{3-}}$ complex. Therefore, the I172A mutation results in a substantial *decrease* in pK_{EHI} compared to that for wildtype *Tbb*TIM.

$$K_i = \left(\frac{K_a}{K_a + [H^+]}\right)(K_i)_{obs} \quad (2)$$

$$K_i = \frac{(K_i)_E\left(1 + \frac{[H^+]}{K_{EH}}\right)}{\left(1 + \frac{[H^+]}{K_{EHI}}\right)} \quad (3)$$

The I172A mutation at *Tbb*TIM results in a 100-fold decrease in k_{cat}/K_m for substrate isomerization (Table S1),[28] which is similar to the 100-fold lower affinity of the mutant compared with the wildtype enzyme for $\mathbf{I^{3-}}$ observed at pH < 6 (Figure 2). Now, if the effect of the I172A mutation on the binding of PGA results from a perturbation of the basicity of Glu-167 at the $\mathbf{EH{\bullet}I^{3-}}$ complex, so that there is a larger thermodynamic driving force for protonation of the $\mathbf{E{\bullet}I^{3-}}$ complex to give $\mathbf{EH{\bullet}I^{3-}}$ for the wildtype enzyme, then the 100-fold higher affinity of the wildtype enzyme for $\mathbf{I^{3-}}$ is consistent with pK_{EHI} ≈ 10 for ionization of Glu-167 at the wildtype $\mathbf{EH{\bullet}I^{3-}}$ complex. The catalytic properties of *Tbb*TIM are similar to those of TIMs from other organisms,[18,26] so that the value of pK_{EH} = 3.9 determined for the side chain of Glu-165 at unliganded wildtype yeast TIM[16] is expected to be similar to that of Glu-167 at unliganded wildtype *Tbb*TIM. Therefore, we conclude that the binding of $\mathbf{I^{3-}}$ to wildtype *Tbb*TIM results in an ~6 pK unit increase in the basicity of the carboxylate side chain of the catalytic base Glu-167, from pK_{EH} ≈ 4 for the free enzyme,[16] to pK_{EHI} ≈ 10 for the $\mathbf{EH{\bullet}I^{3-}}$ complex. We attribute the large increase in the thermodynamic driving force for protonation of the side chain of the catalytic glutamate at the $\mathbf{E{\bullet}I^{3-}}$ complex to give $\mathbf{EH{\bullet}I^{3-}}$ to the combined effects of the relief of destabilizing electrostatic interactions between the neighboring carboxylate anions of TIM and $\mathbf{I^{3-}}$ and the formation of a stabilizing enzyme-inhibitor hydrogen bond.[25]

The closure of loop 6 of TIM over bound ligands is accompanied by movement of the hydrophobic side chain of Ile-172 towards the carboxylate side chain of Glu-167, as the latter side chain swings towards the ligand and the hydrophobic side chain of Leu-232 (Figure 3).[12] This large conformational change converts the inactive open enzyme $\mathbf{E_O}$ to the active closed enzyme $\mathbf{E_C}$ and serves to sequester the catalytic base from interaction with

bulk solvent. It results in "clamping" of the carboxylate anion of Glu-167 between the bulky hydrophobic side chains of Ile-172 and Leu-232. The L232A mutation at *Tbb*TIM results in a surprising 20-fold *increase* in the second-order rate constant for enzyme-catalyzed deprotonation of the truncated substrate glycolaldedyde.[27,28] The observation here that the L232A mutation also results in a 20-fold *increase* in the affinity of the enzyme for $\mathbf{I}^{3-}$ at pH 8.3 (Figure 2) is consistent with the proposal that this mutation results in an ~20-fold *increase* in the concentration of the closed enzyme $\mathbf{E_C}$ relative to the open enzyme $\mathbf{E_O}$, and that the intermediate analog $\mathbf{I}^{3-}$ has a high affinity for the closed enzyme $\mathbf{E_C}$, but a much lower affinity for the open enzyme $\mathbf{E_O}$.[27,28]

We propose that the ~2 p*K* unit higher pK_a of the carboxylate group of Glu-167 at the $\mathbf{EH{\bullet}I}^{3-}$ complex for wildtype compared with I172A mutant *Tbb*TIM results from the "clamping" action of the side chain of Ile-172 that leads to *destabilization* of $\mathbf{E{\bullet}I}^{3-}$ by unfavorable electrostatic interactions between the neighboring carboxylate anions of Glu-167 and bound $\mathbf{I}^{3-}$, and *stabilization* of $\mathbf{EH{\bullet}I}^{3-}$ by the formation of a hydrogen bond between the carboxylate group of $\mathbf{I}^{3-}$ and the protonated side chain of Glu-165/167 (Figure 1). Thus the bulky hydrophobic side chain of Ile-172 restricts the movement of the basic carboxylate side chain of Glu-167 relative to $\mathbf{I}^{3-}$ at $\mathbf{E{\bullet}I}^{3-}$, resulting in an increase in the driving force for protonation to give $\mathbf{EH{\bullet}I}^{3-}$. The I172A mutation then lifts this restriction, allowing separation of the carboxylate anions of the enzyme and bound $\mathbf{I}^{3-}$ and relief of the destabilizing electrostatic interactions (Figures 1 and 3).

The binding to TIM of the enediolate phosphate trianion intermediate of the isomerization reaction (Scheme 1) should result in an increase in the basicity of the carboxylate side chain of Glu-165/167 that is similar to that observed upon the binding of the intermediate analog $\mathbf{I}^{3-}$, because each complex is destabilized by electrostatic interactions between a ligand trianion and an enzyme carboxylate oxyanion that are relieved by protonation of the enzyme. The increase in the pK_a of Glu-165/167 will occur as the α-carbonyl proton is transferred from substrate to Glu-165/167, so that the maximal change in the basicity of this residue will occur upon full proton transfer to form the TIM•enediolate complex.[2] This enhancement of the basicity of the catalytic base at TIM results in an increase in the thermodynamic driving force for deprotonation of enzyme bound substrate compared to the driving force in water, and will make a significant contribution to the enzymatic rate acceleration.

PGA trianion is a less than perfect transition state/intermediate analog. For example, the $\mathbf{EH{\bullet}I}^{3-}$ complex is stabilized by a hydrogen bond between the protonated side chain of Glu-165/167 and $\mathbf{I}^{3-}$ (Figure 1), but this hydrogen bond cannot be present in the transition state for deprotonation of TIM-bound substrate, where the carboxylate anion is in the process of abstracting a substrate proton. Also, the transition state is strongly stabilized by the presence of a hydrogen bond between the imidazole side chain of His-95 and the developing C-1 or C-2 oxyanion (Figure 1).[29,30] If the strength of the hydrogen bond between His-95 and the carboxylate of $\mathbf{I}^{3-}$ at the $\mathbf{EH{\bullet}I}^{3-}$ complex is attenuated by the presence of the additional hydrogen bond between $\mathbf{I}^{3-}$ and the carboxylic acid side chain of Glu-165/167 (Figure 1), then the interaction with His-95 may be less significant for stabilization of the $\mathbf{EH{\bullet}I}^{3-}$ complex than for transition state stabilization.

At pH 4.9, where $K_i = 1.2 \times 10^{-7}$ M for wildtype *Tbb*TIM (Table S2), only ~10% of the enzyme is expected to be present in the protonated **EH** form (p$K_{EH} \approx 4$, Scheme 2). This is consistent with $(K_i)_{EH} \approx 1.2 \times 10^{-8}$ M for breakdown of the $\mathbf{EH{\bullet}I}^{3-}$ complex at pH << 4, where the side chain of Glu-167 at the free enzyme is fully protonated, which corresponds to a binding energy of 11 kcal/mol for formation of $\mathbf{EH{\bullet}I}^{3-}$ from $\mathbf{EH} + \mathbf{I}^{3-}$ (Scheme 2). This binding energy is only ~3 kcal/mol smaller than the total intrinsic transition state binding

energy of 14 kcal/mol estimated for the natural substrate GAP.[31,32] We conclude that the stabilizing electrostatic interactions between TIM and the small two-carbon transition state/intermediate analog $\mathbf{I}^{3-}$ account for a very large fraction of the total transition state stabilization for the TIM-catalyzed reaction.

In conclusion, PGA trianion is a good transition state analog that expresses ~80% of the total intrinsic transition state binding energy for TIM. The binding of PGA trianion at the desolvated enzyme active site results in destabilizing electrostatic interactions of its carboxylate group with the carboxylate side chain of Glu-165/167 and results in a substantial enhancement of the basicity of this catalytic base, which functions to deprotonate enzyme bound substrate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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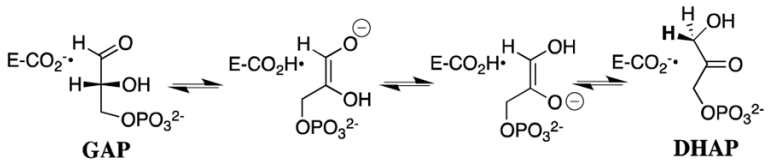
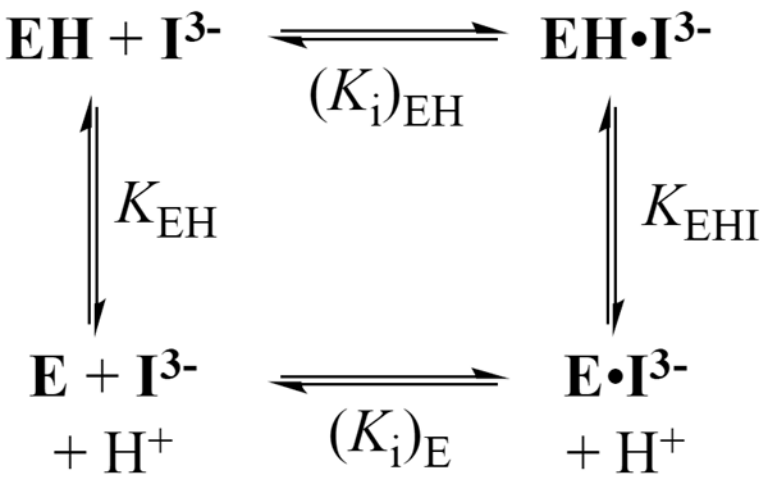


Scheme 1.



Scheme 2.

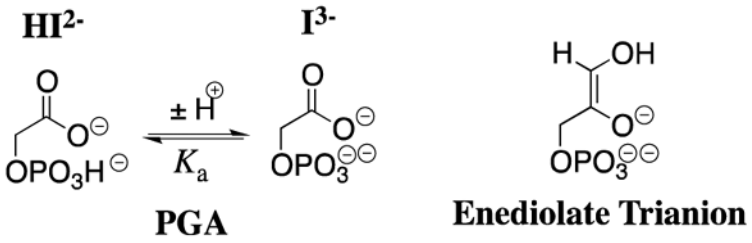


Chart 1.

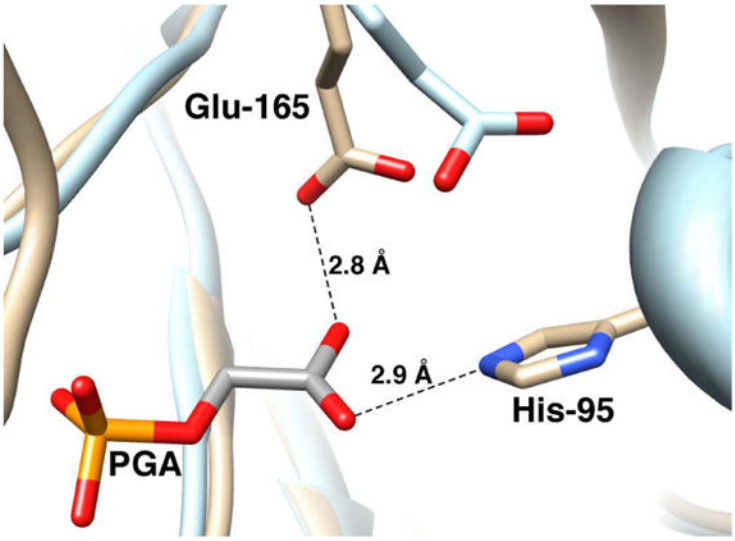


Figure 1.
Models, from X ray crystal structures, of the active site of unliganded yeast TIM (light blue, PDB entry 1YPI) and yeast TIM complexed with PGA (tan, PDB entry 2YPI). Ligand binding is accompanied by a 2 Å shift in the position of the carboxylate side chain of Glu-165 towards the bound ligand. The active sites of TIM from yeast and *Trypanosoma brucei brucei* are virtually superimposable, except that the catalytic base at *Tbb*TIM is at position 167 in the protein sequence.

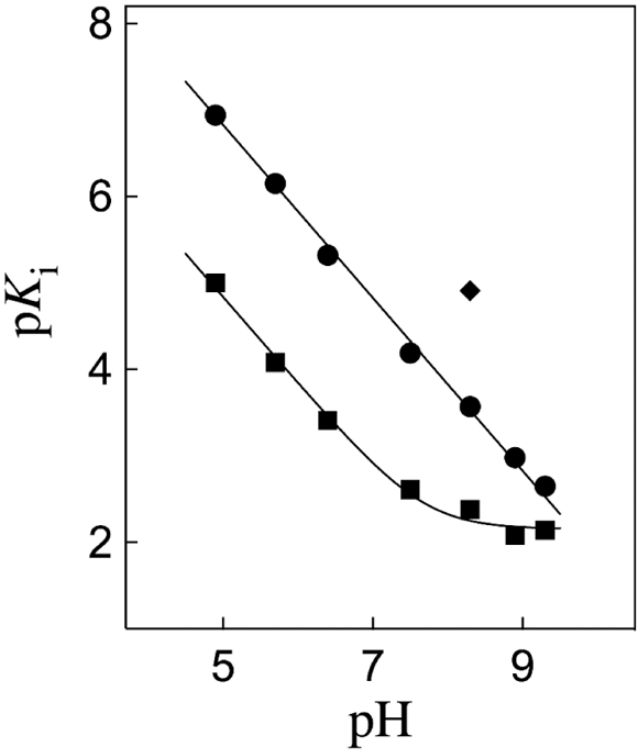


Figure 2.
Dependence on pH of the dissociation constant K_i (M) for breakdown of the complex between $\mathbf{I}^{3-}$ and wildtype *Tbb*TIM (●), I172A mutant *Tbb*TIM (■), and L232A mutant *Tbb*TIM (◆) at 25 °C and I = 0.1 (NaCl).

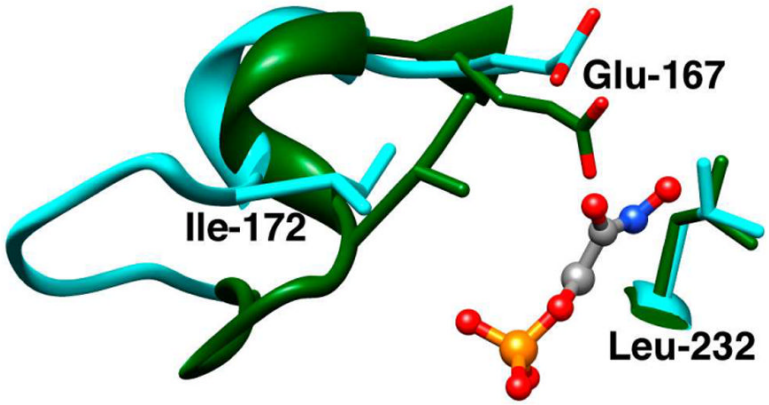


Figure 3.

Models, from X-ray crystal structures, of the active sites of unliganded *Tbb*TIM in the open form (cyan, PDB entry 5TIM) and the closed enzyme liganded by phosphoglycolohydroxamate (green, PDB entry 1TRD). Closure of loop 6 (residues 168 – 178) over bound ligand results in movement of the side chain of Ile-172 towards the side chain of Glu-167 and of this carboxylate side chain towards the side chain of Leu-232.