

T. TRIOSE PHOSPHATE ISOMERASE

Background

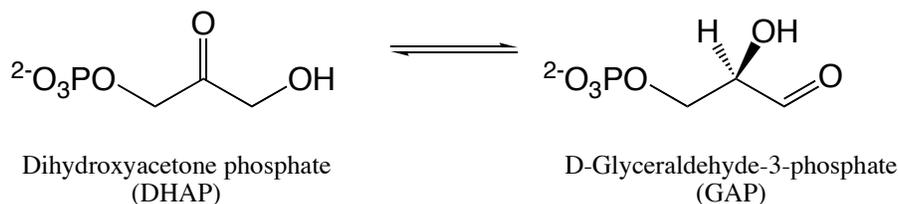


Figure T.1. The reaction catalyzed by triose phosphate isomerase. Note that the reaction can proceed in either direction.

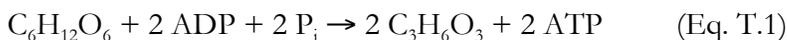
Triose phosphate isomerase (TIM) is one of the best studied enzymes out there. A glycolytic enzyme, TIM is present in virtually all organisms. It catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) with D-glyceraldehyde-3-phosphate (GAP; Figure T.1). The individual principally associated with TIM is Jeremy Knowles, who has written two excellent reviews that cover most of the material in these notes.¹ The structure of TIM was solved by Greg Petsko in the mid-1970's, at a time when relatively few enzyme structures were known. From my perspective, the value of TIM as a point of discussion lies in the following topics:

- General acid/base catalysis
- Moderation of pK_a 's in the enzyme active site
- Sequestration of reactive intermediates
- Optimization of catalytic efficiency
- Cryptic stereochemistry

Each topic will be covered below.

Glycolysis

Glucose is metabolized anaerobically through the glycolytic pathway which uses 11 enzymes to convert glucose to two molecules of lactic acid along with the production of two molecules of ATP from ADP and inorganic phosphate (Eq. T.1)



To obtain a better understanding of glycolysis, any textbook or Wikipedia entry will do. However, to place TIM's role in context, it appears at an important juncture in glycolysis, where the last hexose in the cycle, fructose-1,6-bisphosphate, is degraded to glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP; Figure T.2). Since GAP is the substrate for the next enzyme

¹(a) Knowles (1991) Enzyme catalysis: not different, just better. *Nature* **350**, 121-124 (b) Knowles (1991) To Build an Enzyme... *Phil. Trans. R. Soc. Lond. B* **332**, 115-121.

in the pathway (GAP dehydrogenase), it behooves the organism to convert DHAP to GAP so that none of the original hexose goes to waste. TIM catalyzes the interconversion of DHAP to GAP, so that the cleavage of fructose-1,6-bisphosphate ultimately yields two molecules of GAP for subsequent metabolism (Figure T.2).

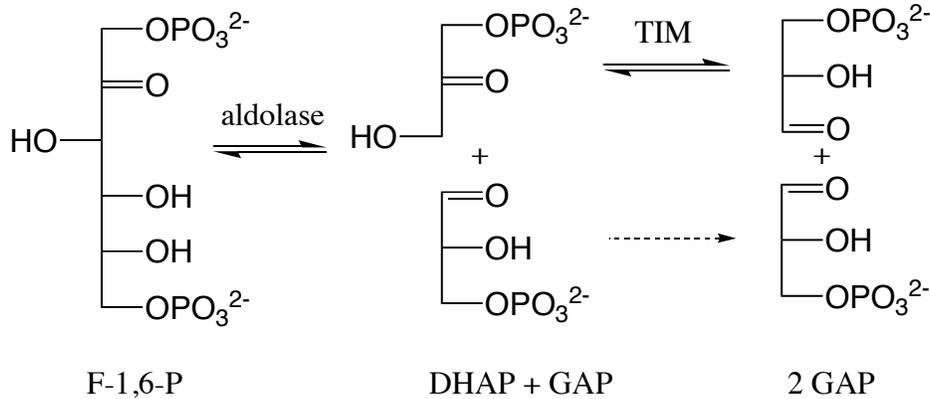


Figure T.2. In the glycolytic pathway, TIM catalyzes the conversion of DHAP to GAP so that the breakdown products of fructose-1,6-bisphosphate (F-1,6-P) are two molecules of GAP, which is the substrate of the next enzyme in the pathway, glyceraldehyde-3-phosphate dehydrogenase.

The equilibrium constant between DHAP and GAP is 0.05 (Eq. T.2), so dihydroxyacetone phosphate is actually preferred at equilibrium. However, GAP is rapidly removed by GAP dehydrogenase in the next step of glycolysis, assuring that the reaction catalyzed by TIM flows essentially in a single direction, from DHAP to GAP, in the cell.



TIM, the Protein

Triose phosphate isomerase is a functional dimer, composed of two identical subunits of approximately 250 residues. The two active sites present in the dimer act independently of each other. The tertiary structure of TIM is composed of a single domain, the so-called “TIM barrel” or $\alpha_8\beta_8$ -barrel. This structure, the most common fold found in enzymes, is composed of eight central β strands that form an infinite β sheet, wrapped in a barrel (Figure T.3). Each pair of strands is connected by an α helix, a subsidiary $\beta\alpha\beta$ motif that is repeated around the cylindrical barrel.

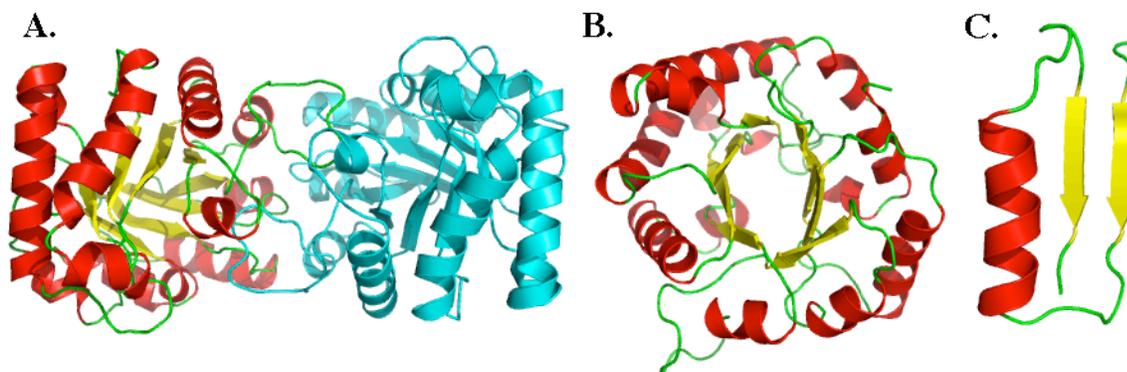


Figure T.3. The structure of TIM. (A) The TIM dimer. The left subunit is colored by secondary structure and the right dimer is colored turquoise. (B) The TIM monomer colored by secondary structure. (C) The β - α - β connection forming the TIM barrel.

The active site of TIM is located in the center of the barrel at the C-terminal end of the β strands. When the substrate is bound, a flexible loop formed by residues 169-176 folds over the active site, isolating the substrate from solution (Figure T.4).

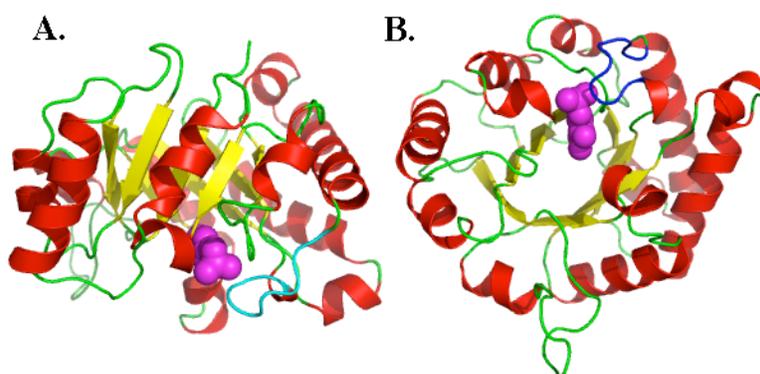


Figure T.4. Substrate binding by TIM. (A) The substrate (colored magenta) binds at the C-terminus of the barrel. (B) View from the bottom. Note loop in dark blue that wraps over substrate.

Kinetics and Chemical Mechanism

A Perfect Enzyme

Table T.1. Kinetic constants for the interconversions of GAP and DHAP catalyzed by TIM. (* K_m of GAP does not account for diol equilibrium).

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
GAP	4300	0.4*	9.1×10^6
DHAP	430	0.97	4.4×10^5

How fast can an enzyme be? The diffusion limit for small molecules reaching the active site of an enzyme has a second order rate constant of 10^8 - 10^9 $M^{-1}s^{-1}$. By comparison, TIM – in converting GAP to DHAP appears to be within that limit, at roughly 10^7 $M^{-1}s^{-1}$ (Table T.1). However, things are a little complicated. Glyceraldehyde-3-phosphate exists in an equilibrium with its diol, and the equilibrium strongly favors the diol by a factor of 20 (Figure T.3). As a result, when the nominal concentration of GAP is 0.4 mM, in actuality the diol is the dominant form, leaving only a small amount (roughly 0.02 mM) of the actual substrate of TIM, the aldehyde. Thus, the value of k_{cat}/K_m for the conversion of GAP to DHAP is closer to 10^8 $M^{-1}s^{-1}$, right up at the diffusion limit. In other words, TIM cannot convert GAP to DHAP any faster than it does. The Knowles laboratory verified TIM's "perfection" through the use of viscosogens in measuring the rate of conversion of GAP to DHAP. By adding compounds that create a more viscous solution, such as glycerol, the rate of diffusion is decreased. The results indicated a linear decrease in the second order rate constant (k_{cat}/K_m) with increasing solution viscosity, as would be predicted for a diffusion-limited reaction.

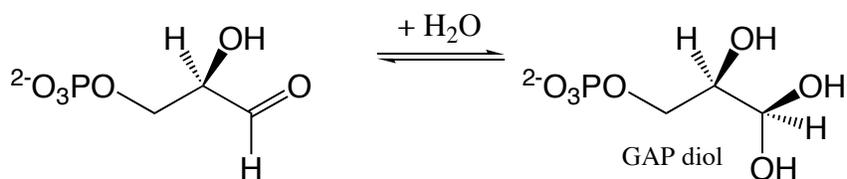


Figure T.3. The hydration of glyceraldehyde-3-phosphate leads to the formation of a diol at C1. The diol is not a substrate for TIM.

Chemical Mechanism

Long ago, two mechanisms were proposed for the transformation of GAP to DHAP. The first, a hydride shift mechanism (Figure T.4A) proceeds by transfer of a hydride equivalent from C1 to C2, passing through a transition state in which both C1 and C2 have substantial sp^2 character. The second mechanism is a proton transfer mechanism, in which deprotonation of GAP leads to the formation of an **enediolate intermediate** which rearranges to form DHAP upon reprotonation (Figure T.4B). The strongest mechanistic evidence for proton transfer came through a chemical exchange experiment (recently updated by J. P. Richard at SUNY Buffalo). When GAP is incubated with TIM in deuterated water, the DHAP formed is roughly 50% deuterated, indicating that there is proton exchange with solvent during the reaction. Since water does not exchange hydride but rather protons, the proton transfer mechanism is indicated. It is noteworthy that the deuterium that does appear on DHAP resides solely in the *pro-R*² position of C1, and some of that deuterated material reacts back to form GAP with a deuterium at C2 (Figure T.5). In addition to supporting the proton transfer mechanism, these results indicate: (a) that the reaction involves a single base transferring protons between C1 and C2, otherwise no retention of hydrogen between GAP and DHAP would be possible, and (b) the reaction proceeds through a *cis*-enediolate intermediate, given the stereochemistry of deuterium incorporation in DHAP and its retention in GAP.

² See Appendix in Lipids notes for discussion of pro-chirality.

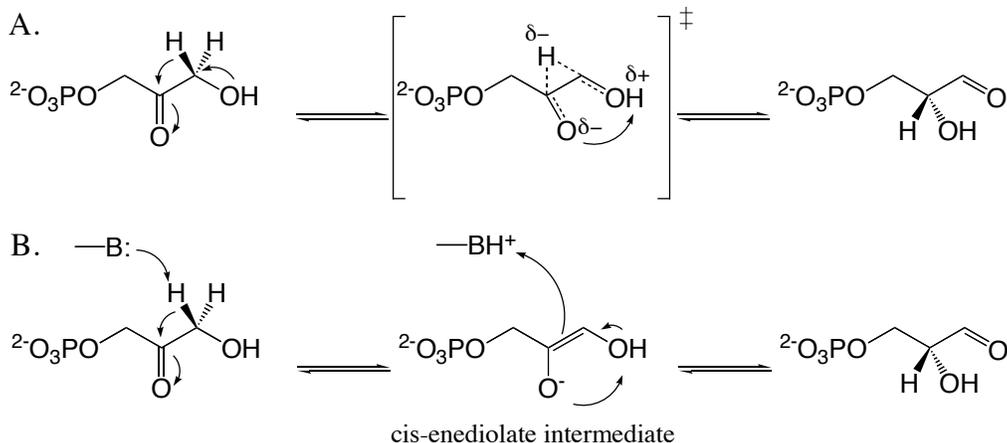


Figure T.4. (A) Hydride shift mechanism from DHAP, via a transition state, to GAP. (B) Proton transfer mechanism via an enediolate intermediate.

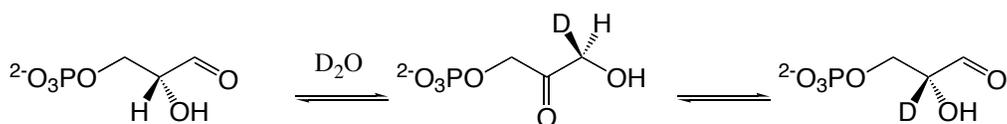


Figure T.5. Deuterium incorporation into DHAP at the *pro*-R position when isomerization of GAP proceeds in D_2O occurs at a rate of about 50%. When the reaction proceeds back to GAP, some of the deuterium label returns to C2.

The presence of a single base in the active site of TIM, capable of exchanging the proton between C1 and C2 of the triose phosphate substrates follows the “rule” of parsimony, which holds that enzyme active sites are designed to use the smallest number of functional residues possible. Since it is presumed to be an evolutionary challenge to place a side chain appropriately for optimum catalytic efficiency, placing two will be more difficult than placing one that can perform dual functions.

The nature of the catalytic mechanism is also evident in the structure of the competitive inhibitors that have been found to act on TIM. Of them, the phosphoglycohydroxamate (Figure T.6) is the most potent, with a K_d of 4 μM . The structure is an excellent mimic of the *cis*-enediolate intermediate which is strongly bound by TIM. Note that a similar inhibitor lacking the double bond character of the hydroxamate and the anionic charge binds over 100 fold more weakly.

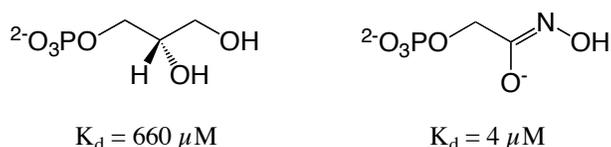


Figure T.6. Competitive inhibitors of TIM.

The identity of the active site base was discovered using a substrate analog, bromohydroxyacetone phosphate (Figure T.7), which contains an extremely reactive electrophile in the acyl bromide functionality. When soaked with TIM, a single residue is covalently modified by this analog, glutamate 165. Importantly, Glu165 is not modified in the presence of the natural substrate,

indicating that Glu165 is the most reactive nucleophile (and therefore the most promising base) in the active site. The “choice” of Glu165 as the active site base is more than somewhat surprising. The side chain carboxylic acid of glutamate has a pK_a of roughly 4. Protons at the α position relative to aldehydes and ketones generally have a pK_a of 19. That implies that the transfer of a proton from one of the triose phosphate substrates to Glu165 will have an equilibrium constant of roughly 10^{-15} !

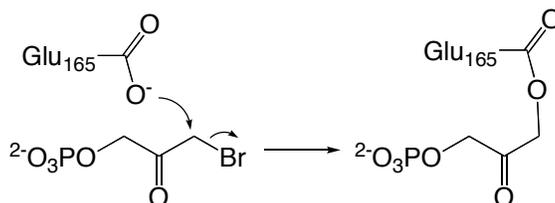


Figure T.7. Bromohydroxyacetone phosphate modifies the active site base, Glu165.

General Acid-Base Catalysis

Residues functioning as active site acids and bases are common in enzyme catalysis. Biochemical transformations are commonly accompanied by a shift in charge distribution within the molecule. In instances where negative charge grows at a given position, it can often be neutralized by transfer of a hydrogen ion, and in instances where positive charge grows, it can be relieved by removal of a proton (Figure T.8). In addition, there are many examples of biochemical transformations in which the transformation itself is dependent on the movement of a hydrogen ion (as with TIM; Figure T.4B). Any time an acid other than H_3O^+ is involved in proton donation during catalysis, it is referred to as **general acid** catalysis. When a base other than HO^- is involved in a proton transfer, it is **general base** catalysis.³

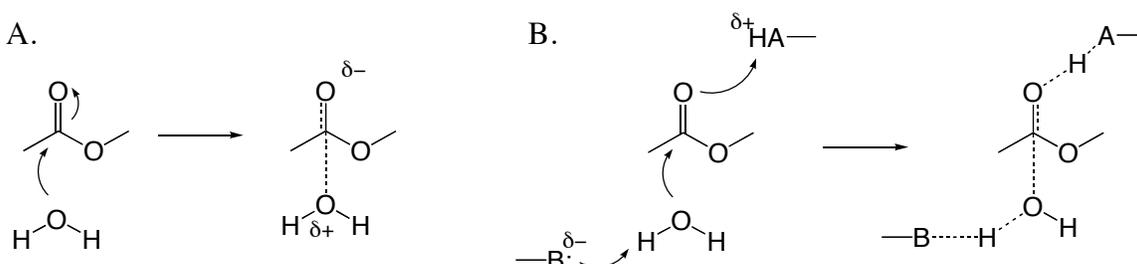


Figure T.8. (A) First step in ester hydrolysis without catalysis. (B) Addition of general base and general acid groups to neutralize growth of charge in transition state.

Biochemical evidence for general acid/base catalysis is often obtained by performing studies of reaction rates vs. pH. If a general acid is required for catalysis, then one will see a drop in activity at high pH as the general acid becomes deprotonated. If a general base is required, then one will see an increase in activity at pH increases due to a deprotonation event that creates the general base. In the case of TIM, just such an increase in activity is observed with pH. A plot of k_{cat} vs. pH for the

³ If hydronium or hydroxide *are* involved, it is **specific** acid/base catalysis.

reaction of DHAP to GAP reveals an increase in activity. This increase can be displayed in one of two plots: (a) the plot of k_{cat} vs. pH, or (b) the plot of $\log_{10}(k_{\text{cat}})$ vs. pH (Figure T.7). The latter plot is particularly useful from a display perspective because a sharp discontinuity between two linear regimes that occurs at the $\text{p}K_a$ of the pH-active species. The reason for the break in the line is due to the algebra related to the fraction of functional protein:

$$\frac{k_{\text{cat}}}{k_{\text{cat}}^{\text{max}}} = \frac{[\text{E}^-]}{[\text{EH}] + [\text{E}^-]} = \frac{K_a[\text{EH}]/[\text{H}^+]}{[\text{EH}] + K_a[\text{EH}]/[\text{H}^+]} = \frac{K_a}{[\text{H}^+] + K_a}$$

where $k_{\text{cat}}^{\text{max}}$ is maximal value of k_{cat} , when the general base is fully deprotonated, yielding the E^- form of the enzyme. At low pH, $[\text{H}^+]$ will be much greater than K_a yielding:

$$\frac{k_{\text{cat}}}{k_{\text{cat}}^{\text{max}}} = \frac{K_a}{[\text{H}^+] + K_a} \approx \frac{K_a}{[\text{H}^+]}$$

$$\log(k_{\text{cat}}) = \log\left(\frac{k_{\text{cat}}^{\text{max}} \cdot K_a}{[\text{H}^+]}\right) = \log(k_{\text{cat}}^{\text{max}} \cdot K_a) + \text{pH}$$

Thus a plot of $\log(k_{\text{cat}})$ vs. pH at low pH will have a slope of 1. At high pH, where K_a is much greater than $[\text{H}^+]$, the following rules apply, yielding a line with a slope of zero and a y-intercept of $\log(k_{\text{cat}}^{\text{max}})$:

$$\frac{k_{\text{cat}}}{k_{\text{cat}}^{\text{max}}} = \frac{K_a}{[\text{H}^+] + K_a} \approx 1$$

$$\log(k_{\text{cat}}) = \log(k_{\text{cat}}^{\text{max}})$$

Note that these two lines will intersect when $\text{pH} = \text{p}K_a$ (an algebra problem that I leave to y'all). In the case of TIM, the inflection occurs at pH 6.2, indicating that the active site general base has a $\text{p}K_a$ of 6.2, an unusually high value for a carboxylate (more on that later). From the plot of $\log(k_{\text{cat}})$ vs pH for TIM it is also clear that there is no general acid present in the active site of TIM with a $\text{p}K_a$ of less than 10 or so.

One can promote the isomerization of DHAP to GAP through the use of a very simple general base, acetate. However, it proves to be a relatively poor substitute for TIM, with a second order rate constant for the acetate-catalyzed reaction of $1 \times 10^{-3} \text{ M}^{-1}\text{s}^{-1}$, over ten orders of magnitude slower than the k_{cat}/K_m value obtained with TIM. Clearly a small shift in the $\text{p}K_a$ of Glu165 is insufficient to achieve that acceleration on its own.

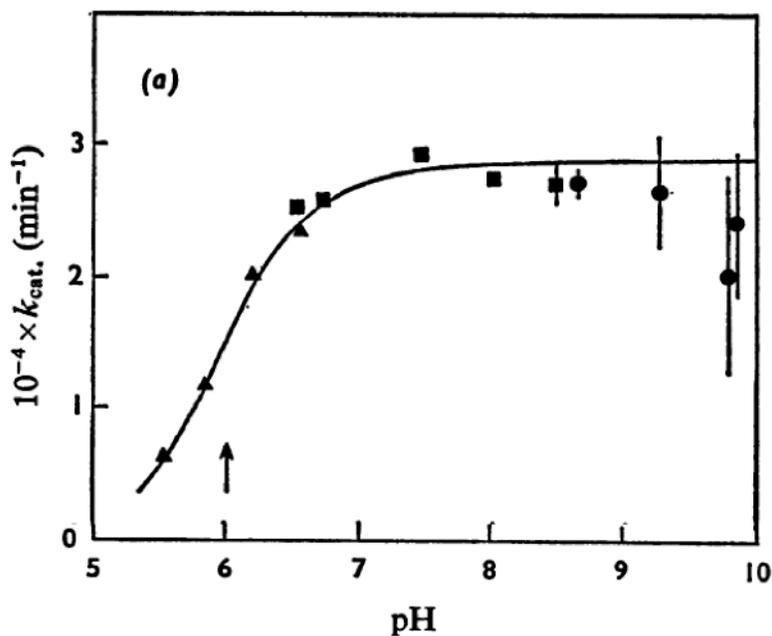


Figure T.8. Plot of $\log(k_{cat})$ vs. pH for TIM.⁴

Structure of the Active Site – Glu165, the General Base

So, back to an interesting question, how does Glu165 manage to relieve a proton from one of the triose phosphate substrates? Part of the answer clearly relates to the alteration in the K_a of the carboxylic acid, which is shifted 100 fold higher than is typical for glutamic acid, yielding a stronger conjugate base at physiological pH. It isn't unusual to see that kind of shift in pK_a . Using acetic acid as a model carboxylic acid, one sees dramatic differences in the acid dissociation constant depending on solvent (Table T.2). The pK_a of an acid (and the pK_b of its conjugate base) are determined in part by the relative stabilization of the two partners, acid and base, in a given environment. In water, the carboxylate is fairly stable, so deprotonation of the acid is not too bad thermodynamically. In DMSO, a more non-polar solvent, the carboxylate is less stable, so deprotonation of the acid is less favored (higher pK_a).

Table T.2 The pK_a of acetic acid in various solvents.

Solvent	pK_a
Water	4.7
Methanol	9.6
Dimethylsulfoxide	12.6

⁴ From Plaut & Knowles (1972) *Biochem. J.* **129**, 311-320.

Inspection of the active site of TIM bound to the inhibitor, PGH (Figure T.6), shows that the carboxylate is indeed in a very non-polar environment, surrounded by a set of hydrophobic side chains including Ile170 (Figure T.9). In addition, the carboxylate of Glu165 is not that far distant from the phosphate group of the substrate, providing further destabilization of the conjugate base (and therefore weakening the acid). The Richard lab undertook a study to measure the protonation state of Glu165 in the presence of the inhibitor phosphoglycolate (PGA), which surprisingly forms a hydrogen bond between the carboxylate of the inhibitor and Glu165, suggesting protonation of Glu165 (Figure T.10). The K_i for PGA increases (and the pK_i decreases) continuously from pH 5 to 9, indicating that **Glu165 is still mostly protonated at pH 9**. If it weren't, the K_i would have plateaued. If Ile170 (Figure T.9) is mutated to alanine, the pK_i levels off at pH 7.5 (Figure T.10), the apparent pK_a of Glu165. Clearly, the active site environment has altered the pK_a of the carboxylate side chain of Glu165 tremendously.

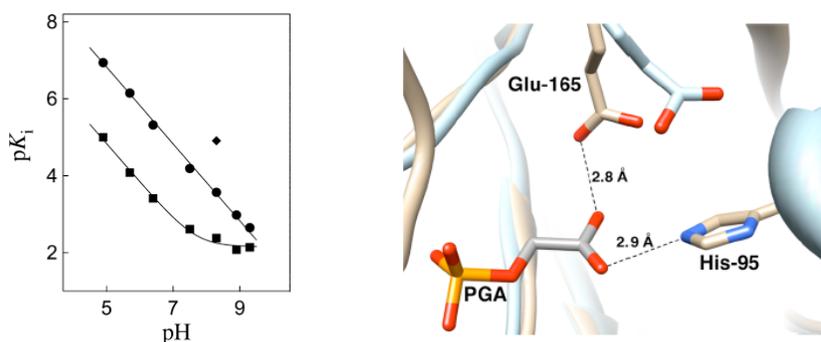


Figure T.10 Effect of pH on inhibition by phosphoglycolate (PGA). The pK_i shows continuous decrease (black circles) with an increase in pH, showing that Glu165 remains largely protonated from pH 5 to 9. The Ile170 to alanine mutant (black squares) shows a pK_a of 7.5 for Glu165, suggesting that the loss of hydrophobicity makes Glu165 a stronger acid.⁵

Structure of the Active Site – His95, the General Acid

Histidine 95 is positioned ideally in the active site of TIM to act as the general acid, despite the fact that there is no downward deflection in the pH activity profile beyond pH 7 (Figure T.8). The structure provides an explanation for that phenomenon (Figure T.11). His95 accepts an H-bond from the backbone amide nitrogen of a residue at the N-terminus of an alpha helix. That H-bond assures that the pK_a of His95 is very low ($\ll 7$) because the conjugate base is effectively stabilized. As a result the putative general acid is not in the ionization state (HisH^+) that one would expect of a general acid. What gives?

Evidence suggests that the neutral form of His95 is acting as the acid during catalysis, protonating the enolate that forms upon proton abstraction (Figure T.4B), which lowers the pK_a of the C-H bond attacked by Glu165. The alkoxide that forms is fairly basic and strong enough to deprotonate a neutral histidine ($pK_a \approx 14$). Likewise, once the enediol collapses back to product, the anionic

⁵ Malabanan et al. (2013) Magnitude and Origin of the Enhanced Basicity of the Catalytic Glutamate of Triosephosphate Isomerase. *J. Am. Chem. Soc.* **135**, 5978-5981.

conjugate base of His95 is strong enough to deprotonate the hydroxyl group ($pK_a \approx 15$) so that rapid proton transfer is allowed.

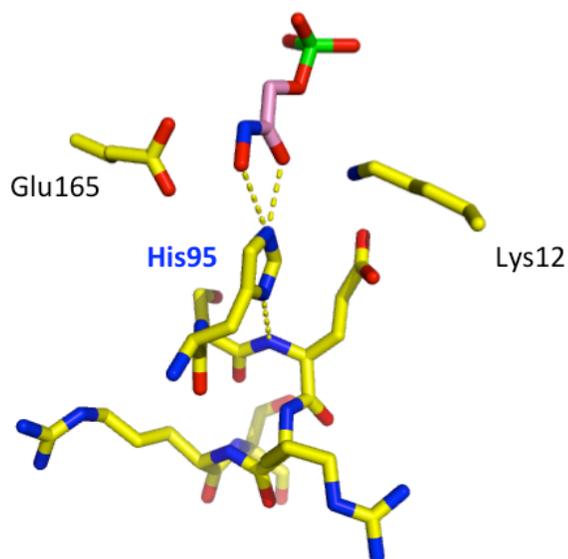


Figure T.11. Active site of TIM with the inhibitor PGH bound. Note that His95 accepts an H-bond from the backbone amide of an adjacent residue (Glu97), which constrains it to the neutral ionization state.