Published in final edited form as:

Curr Opin Chem Biol. 2014 August ; 0: 1–10. doi:10.1016/j.cbpa.2014.03.001.

Enzyme Architecture: On the Importance of Being in a Protein Cage

John P. Richard^{†,*}, Tina L. Amyes[†], Bogdana Goryanova, and Xiang Zhai

[†]Department of Chemistry, University at Buffalo, SUNY, Buffalo, NY 14260-3000, USA.

Abstract

Substrate binding occludes water from the active sites of enzymes. There is a correlation between the burden to enzymatic catalysis of deprotonation of carbon acids and the substrate immobilization at solvent-occluded active sites for ketosteroid isomerase (KSI - small burden, substrate $pK_a = 13$), triosephosphate isomerase (TIM, substrate $pK_a \approx 18$) and diaminopimelate epimerase (DAP epimerase, large burden, substrate $pK_a \approx 29$) catalyzed reaction. KSI binds substrates at a surface cleft, TIM binds substrate at an exposed “cage” formed by closure of flexible loops; and, DAP epimerase binds substrate in a tight cage formed by an “oyster-like” clamping motion of protein domains. Directed evolution of a solvent-occluded active site at a designed protein catalyst of the Kemp elimination reaction is discussed.

INTRODUCTION

Not everything is possible for enzymes [1,2], and the different explanations for their rate accelerations are difficult to sort through. Enzymes exert a high degree of control over the local environment of the bound substrate, and there are interesting proposals about how this control leads to effective catalysis. Our recent work has focused on catalysis by triosephosphate isomerase (TIM, Figure 1A) [3], orotidine 5'-phosphate decarboxylase [4], and isopentenyl diphosphate isomerase [5], through Michaelis complexes where the substrate is locked in a protein cage [6]. There are at least two imperatives for the evolution of water-occluded, *catalytic cages*. First, caged Michaelis complexes allow for a larger number of enzyme-ligand interactions than is possible for binding at enzyme clefts, where part of the ligand remains exposed to water [6]. Second, the transfer of ligands from aqueous solution to a protein cage enhances polar interactions between the enzyme and ligand, because these strengthen with the reciprocal of the medium dielectric constant ($1/D$) [7], which is larger at protein interiors [8,9], compared with water. This essay will review recent results, which highlight advantages for catalysis at water-occluded, highly organized, protein cages compared with the chaotic solvent water.

© 2014 Elsevier Ltd. All rights reserved.

*To whom correspondence should be addressed. jrichard@buffalo.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

On the Importance of Not Being in Water

The polar solvent water provides strong stabilization of the ionic transition states for heterolytic reactions, such as nucleophilic substitution reactions at carbon, in comparison to less polar solvents such as methanol and acetone [10]. This reflects the effective hydrogen bonding/electrostatic interactions between the solvent water and ionic transition states, compared with nonpolar solvents. A consideration more relevant to enzymatic rate enhancements is the medium effect on the relative stability of ground and transition states, for reactions in which there is a redistribution of charge on proceeding to the transition state [11]. This charge redistribution is strongly promoted at organized nonpolar enzyme active sites, through selective destabilization of charge at the reactant complex [ground-state effect] and stabilization of developing charge at the transition state.

Ground-state effects—Water stabilizes charge through hydrogen bonding/electrostatic interactions. The loss of this stabilization by *desolvation* can result in an increase in the driving force for reactions that neutralize the destabilized charge. A classic example is the large increase in the basicity of a carboxylate anion towards deprotonation of carbon, upon transfer of the anion from water to DMSO [12]. The cost of desolvation of the enzyme and substrate may be paid for by the utilization of the substrate binding energy [13]. This binding energy is recovered, as *activation* of the Michaelis complex for the enzyme-catalyzed conversion to product, when the destabilization from desolvation is lost at the transition state for the enzymatic reaction [11,13].

Transition State Effects—The placement of a hydrogen bonding or of an appropriately charged side-chain next to an atom that *gains* charge on proceeding from reactant to transition state will result in the stabilization of the transition state. The strength of these ionic/hydrogen-bonding interactions increases with the reciprocal of the dielectric constant of the reaction medium. They will increase upon the transfer of reactants from water ($D = 79$) to nonpolar enzyme active sites, for which values of D from 6 - 20 have been estimated from comparisons of thermodynamic parameters [*e.g.*, pK_a s and redox potentials] determined for reactions in water and at protein interiors [8,9].

Triosephosphate Isomerase (TIM)

TIM catalyzes the stereospecific and reversible conversion of dihydroxyacetone phosphate (DHAP) to (R)-glyceraldehyde 3-phosphate (GAP), by a proton transfer mechanism through enzyme-bound *cis*-enediolate reaction intermediates (Figure 1A) [3,14-17]. Unliganded TIM exists in an open conformation: the binding of substrate and transition state analogs is accompanied by a complex phosphodianion-driven conformational change to form the caged catalytic complex shown in Figure 1A [14,18]. The ligand-gated conformational change plays a critical role in enabling transition state stabilization for the TIM-catalyzed reaction. Interactions between TIM and the phosphodianion of GAP account for 80% of the enzymatic rate acceleration [19,20], while the binding of phosphite dianion to TIM results in a 4 kcal/mol stabilization of the transition state for the unactivated reaction of glycolaldehyde (GA) [18]. This is not due to a direct stabilizing interaction between the transition state and dianion, because no such interactions are possible [21]. Rather, a large fraction of the intrinsic binding energy of the substrate dianion, or phosphite dianion, is used

to drive the energetically demanding change in enzyme conformation, from an inactive open form (E_O , Figure 2A) to the high-energy enzyme cage (E_C , $K_C \ll 1$), which shows a high reactivity towards deprotonation of GA [3,22]. The creation of this water-occluded cage reduces the thermodynamic barrier for the enolization of α -carbonyl substrates, by providing direct stabilization of the transition state, and by enhancing the basicity of the carboxylate chain of Glu165, which deprotonates the enzyme-bound substrate.

Transition state effects—The formation of the caged Michaelis complex results in extensive desolvation of DHAP and TIM (Figure 2B). The *total* energetic cost of desolvation is not normally considered in discussions of enzymatic rate enhancements, when the stabilizing interactions with water are replaced by hydrogen-bonding and ion-pairing interactions between the enzyme and substrate. The carbonyl group of DHAP undergoes a change in charge from 0 to -1 upon enolization to the *cis*-enediolate. This negative charge is stabilized by interactions with the side chain cation of Lys12 [23] and the neutral imidazole side chain of His95 [24] (Figure 1B). The contribution of these interactions to the catalytic rate acceleration reflects:

- (1) The entropic advantage to formation of the *cis*-enediolate oxyanion at an organized enzyme active site. This arises from the elimination of the energetic price for reorganization of the water dipoles that occurs for reactions in aqueous solution [25,26]. The cationic side chain of K12 lies at the protein surface (Figure 1A) and is held next to the substrate phosphodianion by a hydrogen bond to the carboxylate side chain of E97 [27]. The latter side chain assists in the rescue of the catalytic activity of K12G mutant TIM by small alkyl ammonium cations [28]. The side chain of H95 is locked in the tightly packed interior of TIM (Figure 1B).
- (2) The greater acidity of the imidazole side-chain, compared with water; the advantage for the ion-pairing interaction of the transition state with the K12 side chain cation; and, the strengthening of all polar interactions at the caged complex, compared with water, due to the smaller effective dielectric constant of the enzyme active site.

Ground-state effects—The six water molecules that lie within 5 Å of the basic side chain of Glu167 for TIM from *Trypanosoma brucei brucei* (Figure 1C) [29], is the same number of molecules observed in the first solvation shell of acetate anion in water [30]. An unperturbed pK_a of 3.9 has been determined for the catalytic side chain at yeast TIM [31]. The formation of the caged Michaelis complex results in partial desolvation of the catalytic carboxylate anion - the side chain of TIM (*Leishmania mexicana*) at the TIM-phosphoglycolohydroxamate complex lies within hydrogen bonding distance of only two waters (Figure 1D) [32]. This conformational change is accompanied by movement of the side chain of Ile172 toward the carboxylate side chain of Glu167 (Glu165 for TIM from yeast and chicken muscle), and of this basic side chain towards the side chain of Leu232, whose position remains fixed (Figure 1E). These motions act to sandwich the partly desolvated $-COO^-$ from Glu167 between the hydrophobic side-chains of Ile172 and Leu232 [33].

The difference between the observed substrate binding energy [$(\Delta G_B)_{\text{obs}}$] and the intrinsic substrate binding energy for desolvated TIM [$(\Delta G_B)_{\text{int}}$], is the energy utilized to drive the desolvation of TIM [$(\Delta G_B)_{\text{desol}}$, Figure 2C]. Recent results provide evidence that the energetic cost of side chain desolvation is recovered as an enhancement in side chain basicity [13]. Phosphoglycolate trianion (**PGA**), an analog of the *cis*-enediolate trianion (Figure 2D), shows a high affinity for binding to TIM [34]. The complex between **PGA** and TIM is stabilized by a hydrogen bond between the carboxylic acid side chain of Glu167 and the inhibitor carboxylate anion (Figure 1F). The $pK_a > 10$ for the carboxylic acid side chain of Glu167 at the inhibitor complex is > 6 units larger than the side chain pK_a at free TIM [35]. This perturbation in side-chain pK_a is due to stabilization of the protonated side chain by the hydrogen bond to **PGA**, and to destabilization of the ionized enzyme by electrostatic interactions between the immobilized enzyme and inhibitor carboxylate anions (Figure 1F). The I172A mutation at *Tbb*TIM results in a 100-fold decrease in k_{cat}/K_m for isomerization [36]; a 100-fold decrease in the affinity of the transition state analog **PGA** at pH 7 [35]; and, a decrease from > 10 to 7.7 in the pK_a of the side chain of Glu167 at the inhibitor complex (Figures 1E and 1F) [35]. This shows that the hydrophobic side chain of Ile172 plays a role in enhancing the basicity of the carboxylate side chain of Glu167.

The contribution to the enzymatic rate enhancement of the presumably large increase, from $pK_a \approx 4$ for free TIM to $pK_a > 10$ for the *cis*-enediolate phosphate trianion intermediate, in the basicity of the carboxylate side chain of Glu165/167 will depend upon the extent to which this increase in pK_a is expressed at the transition state for TIM-catalyzed deprotonation. A maximum rate enhancement will be observed at a late product-like transition state [38]. Similarly, the carboxylate side chain of Glu461 acts as a Brønsted base in catalysis of the transfer of a covalent β -galactosyl reaction intermediate from β -galactosidase to alcohol nucleophiles [37,39]. A related enhancement in the *basicity* of this side chain, on proceeding from the covalent intermediate to the transition state for β -galactosyl group transfer, has been proposed to make a large contribution to the rate enhancement for β -galactosidase [38,40,41].

3-Oxo- Δ^5 -steroid isomerase (KSI) catalyzes the double bond migration reaction shown in Figure 3A, through a delocalized carbanion reaction intermediate. The thermodynamic barrier to deprotonation of the steroid carbon acid ($pK_a = 13$, [42]) is 7 kcal/mol smaller than for deprotonation of DHAP ($pK_a = 18$, [20]), so that the burden borne by KSI [20,42-44] is smaller than for TIM [45]. The rate enhancement for KSI is achieved at an active site situated in a shallow cleft on the enzyme, and using interactions with a single substrate face (Figure 3A) [46]. This suggests that evolution of the more complex architecture for TIM, and the use of mobile loops to create an enzyme-substrate cage (Figure 1A) [14,18], was enforced by TIM's larger catalytic burden and the resulting requirement for stronger stabilizing enzyme-transition state interactions [6].

Ligand binding at the active site cleft of KSI occludes water – for example, the multiple rings of the inhibitor equilenin prevent water from approaching within 10 Å of the protonated side-chains of Tyr16 and Asp103 (numbering for the enzyme from *Pseudomonas putida*), which form stabilizing hydrogen bonds to the enolate intermediate [43,46]. These hydrogen bonds (Figure 3A) are estimated to provide a 4 kcal/mol larger stabilization of the

transition state for isomerization at the enzyme active site compared with isomerization in water. This reflects the preorganization of the side chains at the enzyme active site [25,47], and the strengthening of polar hydrogen bonding interactions at the nonpolar enzyme active site [48]. Ligand-driven desolvation of the active site will enhance the basicity of the side-chain of Asp40. There is evidence that this side chain is immobilized and properly positioned at the enzyme active site by *anion-aromatic* interactions with the phenyl side chains of Phe54 and Phe116 [49] (numbering for the enzyme from *Comamonas testosterone*).

Amino acid Racemases

The 11 unit difference in the pK_a for deprotonation of the α -carbonyl carbon of DHAP ($pK_a = 18$ [20]) and of the α -NH₃⁺ carbon of the zwitterionic form of glycine [50] or proline [51] ($pK_a = 29$) corresponds to a 15 kcal/mol larger thermodynamic barrier for the latter reactions. This larger thermodynamic barrier is only partly balanced by the smaller intrinsic reaction barrier for deprotonation of amino acids [50,52], so that the burden borne by enzymes such as diaminopimelate (DAP) epimerase (Figure 3C) [53,54], glutamate racemase [55], and proline racemase [56] is much larger than for TIM [45]. The origin of the 19 kcal/mol transition state binding energy for proline racemase-catalyzed deprotonation of proline zwitterion [51] was considered several years ago, when it was noted that the change in reaction medium from water to the gas phase should result a 100 kcal/mol stabilization of the intramolecular (internal) charges at the zwitterionic carbanion, compared with intermolecular charges at the reacting anionic base and N-protonated carbon acid [50,51,57]. The less dramatic, but related, change in reaction medium from water to a nonpolar enzyme active site was proposed to result in significant stabilization of N-protonated amino acid carbanion zwitterions at enzyme cages [50,51,57]. This proposal was supported by a computational study on the importance of being zwitterionic, for the racemization catalyzed by glutamate racemase [55].

X-ray crystal structures of DAP epimerase show a striking architecture, which satisfies the imperatives for catalysis of the thermodynamically unfavorable proton transfer [53,54]. The enzyme has a two-domain structure, with C- and N-terminal domains that each contains eight β - and two α strands. The domains are well separated in the unliganded enzyme, where the active site is exposed to water (Figure 3D1). Ligand binding is accompanied by the “oyster shell-like” [54] domain clamping, observed for DAP epimerase modified by the reaction of a basic catalytic thiol with the aziridine substrate analog L,L-azi-DAP (Figure 3D2). The 18° rotation of the N-terminal relative to the C-terminal domain displaces water from the active site cleft and creates a 15 Å long cylindrical active-site cage. The caged-ligand is shown in Figure 3D3, by exposing the enzyme active site. There is a requirement for the precise placement of catalytic side chains at the active site. However, precision in placement, alone, should not cause a large decrease in the thermodynamic barrier to substrate deprotonation, which constitutes a major portion of the barrier to the nonenzymatic reaction. We have proposed that stabilization of the carbanion zwitterion, which results from the reduction in the dielectric constant at the nonpolar enzyme cage, makes a major contribution to the enzymatic rate acceleration [50,51,57].

Designed Protein Catalysts

A common design strategy is to include a hydrophobic aromatic handle at the substrate, which favors transfer from water to greasy active sites. Studies on the retro-aldol cleavage reaction of methodol catalyzed by a designed retroaldolase (Figure 4A) show that the catalytic power, above that observed for catalysis by a simple primary amine, is mostly derived from the strong interactions with the greasy naphthyl ring [58,59]. Similarly, catalysis of the Kemp elimination reaction of 5-nitrobenzisoxazole (Figure 4B) by bovine serum albumin involves binding of the aromatic substrate at a greasy protein cleft close to a catalytic base [60].

Early efforts to design a Kemp eliminase produced a protein of modest activity [61], which has been improved by combining computational design and selection of active catalysts from pools of random mutants [62,63]. Directed evolution resulted in an increase in the kinetic parameters of a designed enzyme from $k_{\text{cat}} = 0.7 \text{ s}^{-1}$, $K_{\text{m}} = 0.0016 \text{ M}$ and $k_{\text{cat}}/K_{\text{m}} = 430 \text{ M}^{-1} \text{ s}^{-1}$ (HG3 [62]) to $k_{\text{cat}} = 7000 \text{ s}^{-1}$, $K_{\text{m}} = 0.0030 \text{ M}$ and $k_{\text{cat}}/K_{\text{m}} = 230,000 \text{ M}^{-1} \text{ s}^{-1}$ (HG3.17 [63]). The X-ray crystal structure of HG3.17 complexed to the inhibitor 6-nitrobenzotriazole provides insight into origin of this rate enhancement (Figure 4C)

(1) Approximately 95% of the ligand surface is buried in a solvent occluded protein cavity. The *evolved* catalyst HG3.17 shows an increased shape complementarity to the substrate analog, but a 2-fold larger K_{m} , so that the enhanced shape complementarity is not reflected by a higher ligand affinity. Directed evolution eliminates a nonproductive binding mode. This should result in equal *increases* in k_{cat} and K_{m} and no change in $k_{\text{cat}}/K_{\text{m}}$, which provides a possible explanation for the weakened ligand binding to HG3.17.

(2) The rate enhancement for HG3.17 compared with HG3 is due to a 3.4 kcal/mol increase in stabilizing protein-ligand interactions, which develop specifically at the transition state. The enhanced transition state stabilization is partly or entirely due to the emergence of a *new* interaction, between the precisely placed amide side chain of Gln50 and the negative charge that develops at the leaving group oxygen (Figure 4C).

(3) Stabilizing interactions, which result from enhanced shape complementarity, may be balanced by an increased cost for desolvation of the protein during substrate binding [ground-state effect]. The price for desolvation of the carboxylate side chain of Asp127, which sits at the bottom of a hydrophobic binding pocket, could then be recovered as an enhancement in side-chain basicity [38]. Directed evolution has no effect on the side-chain basicity of the unliganded catalyst, but this does not exclude an enhanced basicity at the Michaelis complex [63].

(4) The *syn* orbital of a carboxylate oxygen is thought to be more basic than the *anti* orbital [64], but the weakly basic *anti* orbital of the carboxylate side chain of Asp127 is presented to the substrate for catalysis of the Kemp elimination [63]. This suggests that an additional rate enhancement would be obtained for a catalyst that presents the more basic *syn* lone pair to the acidic substrate carbon.

We expect that the design of protein catalysts in which the solvent-occluded active site lies at the bottom or a deep, rigid, protein cavity (Figure 4C) will prove less challenging than the design of catalysts similar to TIM (Figure 1A) of DAP epimerase (Figure 3D), where a solvent-occluded protein cage is created in a ligand-driven conformational change. There may be certain catalytic advantages for active site cages, compared with active site cavities, but these are beyond the scope of this essay.

Summary

The structured microenvironment of substrates bound to enzyme active sites bear little resemblance to the chaotic *structure* of substrates dissolved in water. Several features of these microenvironments favor effective enzymatic catalysis of polar reactions.

- (1) The nonpolar environment of the protein catalyst will enhance all polar interactions, relative to the interactions in water, because the strength of these interactions increases with the reciprocal of the effective local dielectric constant.
- (2) When there is reorganization of charge on moving from the Michaelis complex to the transition state, the substrate is held in a conformation that favors this charge reorganization through stabilization of charge that develops at the transition state.
- (3) The Michaelis complex may be destabilized by desolvation of the enzyme and/or substrate at an active site of low effective dielectric constant, provided this results in a decrease in the barrier for conversion of the bound substrate to the reaction transition state. This is the case when the energetic cost of desolvation of the transition state is *smaller* than that for desolvation of reactants [13].
- (4) The reaction between two ionic molecules of opposite charge to form a product in which the charges are located on a zwitterion is favored by a decrease in the dielectric constant of reaction medium [50]. Amino acid racemases and epimerases catalyze the formation of destabilized carbanion zwitterions at nonpolar active sites, where the thermodynamic reaction barrier is reduced in comparison to the barrier in water [53-55,57].

Acknowledgments

We acknowledge the NIH (GM39754) for generous support of our work described in this review.

REFERENCES

1. Kellett WF, Brunk E, Desai BJ, Fedorov AA, Almo SC, Gerlt JA, Rothlisberger U, Richards NGJ. Computational, Structural, and Kinetic Evidence That *Vibrio vulnificus* FrsA Is Not a Cofactor-Independent Pyruvate Decarboxylase. *Biochemistry*. 2013; 52:1842–1844. [PubMed: 23452154] • The demonstration that, as yet no cofactor-independent pyruvate decarboxylase has been identified. Reports of this activity will be viewed as suspect by adherents to the Pauling model, because the small substrate pyruvate does not appear to provide the large binding energy required for stabilization of the decarboxylation transition state.
2. Lee K-J, Jeong C-S, An YJ, Lee H-J, Park S-J, Seok Y-J, Kim P, Lee J-H, Lee K-H, Cha S-S. FrsA functions as a cofactor-independent decarboxylase to control metabolic flux. *Nature Chem. Biol.* 2011; 7:434–433. [PubMed: 21623357]

3. Richard JP. A Paradigm for Enzyme-Catalyzed Proton Transfer at Carbon: Triosephosphate Isomerase. *Biochemistry*. 2012; 51:2652–2661. [PubMed: 22409228] • An update on the results of more than fifty years of work to determine the mechanism for this seemingly simple enzyme-catalyzed proton transfer reaction.
4. Spong K, Amyes TL, Richard JP. Enzyme Architecture: The Activating Oxyanion Binding Domain for Orotidine 5'-Monophosphate Decarboxylase. *J. Am. Chem. Soc.* 2013; 135:18343–18346. [PubMed: 24274746]
5. Jonnalagadda V, Toth K, Richard JP. Isopentenyl Diphosphate Isomerase Catalyzed Reactions in D₂O: Product Release Limits the Rate of This Sluggish Enzyme-Catalyzed Reaction. *J. Am. Chem. Soc.* 2012; 134:6568–6570. [PubMed: 22471428]
6. Wolfenden R. Enzyme catalysis: Conflicting requirements of substrate access and transition state affinity. *Mol. and Cell. Biochem.* 1974; 3:207–211. [PubMed: 4365214]
7. Hine, J. Structural Effects on Equilibria in Organic Chemistry. Wiley-Interscience; New York: 1975.
8. Kukic P, Farrell D, McIntosh LP, García-Moreno EB, Jensen KS, Toleikis Z, Teilum K, Nielsen JE. Protein Dielectric Constants Determined from NMR Chemical Shift Perturbations. *J. Am. Chem. Soc.* 2013; 135:16968–16976. [PubMed: 24124752] • A novice-friendly discussion of the significance of the different values for effective protein dielectric constants obtained from NMR chemical shift data for atoms at the protein interior, and from thermodynamic data for medium-dependent reactions that occur in the protein interior.
9. Karp DA, Gittis AG, Stahley MR, Fitch CA, Stites WE, García-Moreno B. High Apparent Dielectric Constant Inside a Protein Reflects Structural Reorganization Coupled to the Ionization of an Internal Asp. *Biophys. J.* 2007; 92:2041–2053. [PubMed: 17172297]
10. Grunwald E, S. W. The correlation of solvolysis rates. *J. Am. Chem. Soc.* 1948; 70:846–854.
11. Amyes TL, Richard JP. Specificity in transition state binding: The Pauling model revisited. *Biochemistry*. 2013; 52:2021–2035. [PubMed: 23327224] •• A review which emphasizes that an understanding of the mechanism for stabilization of enzyme-bound transition states is essential for an understanding of enzymatic rate enhancements. Mechanisms for the utilization of nonreacting substrate fragments in transition state stabilization are emphasized.
12. Bordwell FG. Equilibrium acidities in dimethyl sulfoxide solution. *Acc. Chem. Res.* 1988; 21:456–463.
13. Jencks WP. Binding energy, specificity, and enzymic catalysis: the Circe effect. *Adv. Enz. Relat. Areas Mol. Biol.* 1975; 43:219–410.
14. Wierenga RK, Kapetaniou EG, Venkatesan R. Triosephosphate isomerase: a highly evolved biocatalyst. *Cell. Mol. Life Sci.* 2010; 67:3961–3982. [PubMed: 20694739]
15. Knowles JR. Enzyme catalysis: not different, just better. *Nature*. 1991; 350:121–124. [PubMed: 2005961]
16. O'Donoghue AC, Amyes TL, Richard JP. Hydron Transfer Catalyzed by Triosephosphate Isomerase. Products of Isomerization of (R)-Glyceraldehyde 3-Phosphate in D₂O. *Biochemistry*. 2005; 44:2610–2621. [PubMed: 15709774]
17. O'Donoghue AC, Amyes TL, Richard JP. Hydron Transfer Catalyzed by Triosephosphate Isomerase. Products of Isomerization of Dihydroxyacetone Phosphate in D₂O. *Biochemistry*. 2005; 44:2622–2631. [PubMed: 15709775]
18. Malabanan MM, Amyes TL, Richard JP. A role for flexible loops in enzyme catalysis. *Curr. Opin. Struct. Biol.* 2010; 20:702–710. [PubMed: 20951028]
19. Amyes TL, O'Donoghue AC, Richard JP. Contribution of phosphate intrinsic binding energy to the enzymatic rate acceleration for triosephosphate isomerase. *J. Am. Chem. Soc.* 2001; 123:11325–11326. [PubMed: 11697989]
20. Richard JP. Acid-base catalysis of the elimination and isomerization reactions of triose phosphates. *J. Am. Chem. Soc.* 1984; 106:4926–4936.
21. Zhai X, Amyes TL, Wierenga RK, Loria JP, Richard JP. Structural Mutations That Probe the Interactions between the Catalytic and Dianion Activation Sites of Triosephosphate Isomerase. *Biochemistry*. 2013; 52:5928–5940. [PubMed: 23909928]

22. Go MK, Amyes TL, Richard JP. Hydron Transfer Catalyzed by Triosephosphate Isomerase. Products of the Direct and Phosphite-Activated Isomerization of [1-¹³C]-Glycolaldehyde in D₂O. *Biochemistry*. 2009; 48:5769–5778. [PubMed: 19425580]
23. Go MK, Koudelka A, Amyes TL, Richard JP. Role of Lys-12 in Catalysis by Triosephosphate Isomerase: A Two-Part Substrate Approach. *Biochemistry*. 2010; 49:5377–5389. [PubMed: 20481463]
24. Komives EA, Chang LC, Lolis E, Tilton RF, Petsko GA, Knowles JR. Electrophilic catalysis in triosephosphate isomerase: the role of histidine-95. *Biochemistry*. 1991; 30:3011–3019. [PubMed: 2007138]
25. Kamerlin SC, Chu ZT, Warshel A. On catalytic preorganization in oxyanion holes: highlighting the problems with the gas-phase modeling of oxyanion holes and illustrating the need for complete enzyme models. *J. Org. Chem.* 2010; 75:6391–6401. [PubMed: 20825150]
26. Kamerlin S, Sharma P, Chu Z, Warshel A. Ketosteroid isomerase provides further support for the idea that enzymes work by electrostatic preorganization. *Proc. Nat. Acad. Sci.* 2010; 107:4075–4080. [PubMed: 20150513] • A forceful case that electrostatic preorganization makes an important contribution to the rate enhancement for KSI.
27. Samanta M, Murthy MRN, Balam H, Balam P. Revisiting the Mechanism of the Triosephosphate Isomerase Reaction: The Role of the Fully Conserved Glutamic Acid 97 Residue. *Chembiochem*. 2011; 12:1886–1895. [PubMed: 21671330]
28. Go MK, Amyes TL, Richard JP. Rescue of K12G Triosephosphate Isomerase by Ammonium Cations: The Reaction of an Enzyme in Pieces. *J. Am. Chem. Soc.* 2010; 132:13525–13532. [PubMed: 20822141]
29. Wierenga RK, Noble MEM, Vriend G, Nauche S, Hol WGJ. Refined 1.83 Å structure of trypanosomal triosephosphate isomerase crystallized in the presence of 2.4 M-ammonium sulfate. A comparison with the structure of the trypanosomal triosephosphate isomerase-glycerol-3-phosphate complex. *J. Mol. Biol.* 1991; 220:995–1015. [PubMed: 1880808]
30. Alagona G, Ghio C, Kollman P. Monte Carlo simulation studies of the solvation of ions. 1. Acetate anion and methylammonium cation. *J. Am. Chem. Soc.* 1986; 108:185–191.
31. Hartman FC, LaMuraglia GM, Tomozawa Y, Wolfenden R. The influence of pH on the interaction of inhibitors with triosephosphate isomerase and determination of the pK_a of the active-site carboxyl group. *Biochemistry*. 1975; 14:5274–5279. [PubMed: 47]
32. Alahuhta M, Wierenga RK. Atomic Resolution crystallography of a complex of triosephosphate isomerase with a reaction intermediate analog: new insight in the proton transfer reaction mechanism. *Proteins: Struct, Funct, Bioinf.* 2010; 78:1878–1888.
33. Kursula I, Wierenga RK. Crystal structure of triosephosphate isomerase complexed with 2-phosphoglycolate at 0.83-Å resolution. *J. Biol. Chem.* 2003; 278:9544–9551. [PubMed: 12522213]
34. Wolfenden R. Analog approaches to the structure of the transition state in enzyme reactions. *Acc. Chem. Res.* 1972; 5:10–18.
35. Malabanan MM, Nitsch-Velasquez L, Amyes TL, Richard JP. Magnitude and origin of the enhanced basicity of the catalytic glutamate of triosephosphate isomerase. *J. Am. Chem. Soc.* 2013; 135:5978–5981. [PubMed: 23560625] •• An estimate of the effect on pK_a of the conjugate acid, of ligand-gated desolvation of the catalytic carboxylate base of TIM.
36. Malabanan MM, Koudelka AP, Amyes TL, Richard JP. Mechanism for Activation of Triosephosphate Isomerase by Phosphite Dianion: The Role of a Hydrophobic Clamp. *J. Am. Chem. Soc.* 2012; 134:10286–10298. [PubMed: 22583393]
37. Gebler JC, Aebersold R, Withers S. Glu-537, Not Glu-461, Is the Nucleophile in the Active Site of (*lac Z*) β-Galactosidase from *Escherichia coli*. *J. Biol. Chem.* 1992; 267:11126–11130. [PubMed: 1350782]
38. Richard JP. The Enhancement of Enzymic Rate Accelerations by Bronsted Acid-Base Catalysis. *Biochemistry*. 1998; 37:4305–4309. [PubMed: 9556344]
39. Richard JP, Huber RE, Lin S, Heo C, Amyes TL. Structure-reactivity relationships for β-galactosidase (*Escherichia coli*, *lac Z*). 3. Evidence that Glu-461 participates in Bronsted acid-base

- catalysis of β -D-galactopyranosyl group transfer. *Biochemistry*. 1996; 35:12377–12386. [PubMed: 8823173]
40. Richard JP, Huber RE, Heo C, Amyes TL, Lin S. Structure-reactivity relationships for β -galactosidase (*Escherichia coli*, lac Z). 4. Mechanism for reaction of nucleophiles with the galactosyl-enzyme intermediates of E461G and E461Q β -galactosidases. *Biochemistry*. 1996; 35:12387–12401. [PubMed: 8823174]
 41. McIntosh LP, Hand G, Johnson PE, Joshi MD, Körner M, Plesniak LA, Ziser L, Wakarchuk WW, Withers SG. The pKa of the General Acid/Base Carboxyl Group of a Glycosidase Cycles During Catalysis: A ^{13}C -NMR Study of *Bacillus circulans* Xylanase. *Biochemistry*. 1996; 35:9958–9966. [PubMed: 8756457]
 42. Hawkinson DC, Eames TC, Pollack RM. Energetics of 3-oxo -5-steroid isomerase: source of the catalytic power of the enzyme. *Biochemistry*. 1991; 30:10849–10858. [PubMed: 1932007]
 43. Schwans JP, Kraut DA, Herschlag D. Determining the catalytic role of remote substrate binding interactions in ketosteroid isomerase. *Proc. Nat. Acad. Sci.* 2009; 106:14271–14275. [PubMed: 19706511]
 44. Wolfenden R, Snider MJ. The Depth of Chemical Time and the Power of Enzymes as Catalysts. *Acc. Chem. Res.* 2001; 34:938–945. [PubMed: 11747411]
 45. Richard JP, Amyes TL. Proton transfer at carbon. *Curr. Opin. Chem. Biol.* 2001; 5:626–633. [PubMed: 11738171]
 46. Kim SW, Cha SS, Cho HS, Kim JS, Ha NC, Cho MJ, Joo S, Kim KK, Choi KY, Oh BH. High-resolution crystal structures of -5-steroid 3-ketosteroid isomerase with and without a reaction intermediate analogue. *Biochemistry*. 1997; 36:14030–14036. [PubMed: 9369474]
 47. Kamerlin SC, Sharma PK, Chu ZT, Warshel A. Ketosteroid isomerase provides further support for the idea that enzymes work by electrostatic preorganization. *Proc. Nat. Acad. Sci.* 2010; 107:4075–4080. [PubMed: 20150513]
 48. Pasalic H, Aquino AJA, Tunega D, Haberhauer G, Gerzabek MH, Georg HC, Moraes TF, Coutinho K, Canuto S, Lischka H. Thermodynamic stability of hydrogen-bonded systems in polar and nonpolar environments. *J. Comput. Chem.* 2010; 31:2046–2055. [PubMed: 20127744]
 49. Schwans JP, Sunden F, Lassila JK. Use of anion–aromatic interactions to position the general base in the ketosteroid isomerase active site. *Proc. Nat. Acad. Sci.* 2013; 110:11308–11313. [PubMed: 23798413] • • The latest in an important series of papers that present a structure-activity analysis of the contribution of amino acid side chains to the rate enhancement for ketosteroid isomerase.
 50. Rios A, Amyes TL, Richard JP. Formation and stability of organic zwitterions in aqueous solution: enolates of the amino acid glycine and its derivatives. *J. Am. Chem. Soc.* 2000; 122:9373–9385.
 51. Williams G, Maziarz EP, Amyes TL, Wood TD, Richard JP. Formation and stability of the enolates of N-protonated proline methyl ester and proline zwitterion in aqueous solution: A nonenzymatic model for the first step in the racemization of proline catalyzed by proline racemase. *Biochemistry*. 2003; 42:8354–8361. [PubMed: 12846584]
 52. Richard JP, Amyes TL, Toteva MM. Formation and Stability of Carbocations and Carbanions in Water and Intrinsic Barriers to Their Reactions. *Acc. Chem. Res.* 2001; 34:981–988. [PubMed: 11747416]
 53. Pillai B, Moorthie VA, Van Belkum MJ, Marcus SL, Cherney MM, Diaper CM, Vederas JC, James MNG. Crystal Structure of Diaminopimelate Epimerase from *Arabidopsis thaliana*, an Amino Acid Racemase Critical for L-Lysine Biosynthesis. *J. Mol. Biol.* 2009; 385:580–594. [PubMed: 19013471] • A fascinating comparison between the X-ray crystal structures of the open unliganded form of DAP epimerase, and the caged complex with a substrate analog.
 54. Pillai B, Cherney MM, Diaper CM, Sutherland A, Blanchard JS, Vederas JC, James MNG. Structural insights into stereochemical inversion by diaminopimelate epimerase: an antibacterial drug target. *Proc. Nat. Acad. Sci.* 2006; 103:8668–8673. [PubMed: 16723397]
 55. Puig E, Garcia-Viloca M, Gonzalez-Lafont A, Lluch JM. On the ionization state of the substrate in the active site of glutamate racemase. A QM/MM study about the importance of being zwitterionic. *J. Phys. Chem. A.* 2006; 110:717–725. [PubMed: 16405345]
 56. Rubinstein A, Major DT. Catalyzing Racemizations in the Absence of a Cofactor: The Reaction Mechanism in Proline Racemase. *J. Am. Chem. Soc.* 2009; 131:8513–8521. [PubMed: 19492806]

- An interesting theoretical treatment of the rate enhancement of amino acid racemization catalyzed by proline racemase.
57. Richard JP, Amyes TL. On the importance of being zwitterionic: enzymic catalysis of decarboxylation and deprotonation of cationic carbon. *Bioorg. Chem.* 2004; 32:354–366. [PubMed: 15381401]
 58. Jiang L, Althoff EA, Clemente FR, Doyle L, Rothlisberger D, Zanghellini A, Gallaher JL, Betker JL, Tanaka F, Barbas CF, et al. De Novo Computational Design of Retro-Aldol Enzymes. *Science.* 2008; 319:1387–1391. [PubMed: 18323453]
 59. Lassila JK, Baker D, Herschlag D. Origins of catalysis by computationally designed retroaldolase enzymes. *Proc. Nat. Acad. Sci.* 2010; 107:4937–4942. [PubMed: 20194782]
 60. Hollfelder F, Kirby AJ, Tawfik DS, Kikuchi K, Hilvert D. Characterization of Proton-Transfer Catalysis by Serum Albumins. *J. Am. Chem. Soc.* 2000; 122:1022–1029.
 61. Rthlisberger D, Khersonsky O, Wollacott AM, Jiang L, DeChancie J, Betker J, Gallaher JL, Althoff EA, Zanghellini A, Dym O, et al. Kemp elimination catalysts by computational enzyme design. *Nature.* 2008; 453:190–195. [PubMed: 18354394]
 62. Privett HK, Kiss G, Lee TM, Blomberg R, Chica RA, Thomas LM, Hilvert D, Houk KN, Mayo SL. Iterative approach to computational enzyme design. *Proc. Nat. Acad. Sci.* 2012; 109:3790–3795. [PubMed: 22357762]
 63. Blomberg R, Kries H, Pinkas DM, Mittl PRE, Grtter MG, Privett HK, Mayo SL, Hilvert D. Precision is essential for efficient catalysis in an evolved Kemp eliminase. *Nature.* 2013; 503:418–421. [PubMed: 24132235] •• The end result of elegant and sophisticated efforts at the computational design, followed by directed evolution of a protein with enzyme-like catalytic activity.
 64. Gandour RD. On the Important of Orientation in General Base Catalysis by Carboxylate. *Bioorg. Chem.* 1981; 10:169–176.
 65. Jogl G, Rozovsky S, McDermott AE, Tong L. Optimal alignment for enzymatic proton transfer: structure of the Michaelis complex of triosephosphate isomerase at 1.2- resolution. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100:50–55. [PubMed: 12509510]
 66. Malabanan MM, Amyes TL, Richard JP. Mechanism for Activation of Triosephosphate Isomerase by Phosphite Dianion: The Role of a Ligand-Driven Conformational Change. *J. Am. Chem. Soc.* 2011; 133:16428–16431. [PubMed: 21939233]

Figure 1.

Representations of X-ray crystal structures of TIM. (A) Reaction catalyzed by triosephosphate isomerase (TIM) and the caged complex between yeast TIM and PGA [PDB entry 2YPI]. The residues from the phosphodianion gripper loop are shaded cyan. The ammonium nitrogen (blue) from the side chain of K12 is ion paired to oxygen (red) from the ligand phosphodianion. (B) The active site of yeast TIM (PDB entry 1NEY) [65]. Reprinted with permission from Ref. 23. Copyright 2013 American Chemical Society. (C) The open form of TIM from *Trypanosoma brucei brucei* (TbbTIM), which shows the solvated carboxylate side chain of Glu167 (PDB entry 5TIM). (D) The desolvated closed form of TIM from *Leishmania mexicana* liganded to phosphoglycolohydroxamate (PGH) (PDB entry 2VXN). (E) An overlay of the active sites of unliganded open (cyan, PDB entry 5TIM) and PGH-liganded closed TbbTIM (green, PDB entry 1TRD). Reprinted with permission from Ref. 66. Copyright 2011 American Chemical Society. (F) An overlay of the active sites of unliganded (light blue, PDB entry 1YPI) and the PGA complex (tan, PDB entry 2YPI) of yeast TIM. Reprinted with permission from Ref. 35. Copyright 2013 American Chemical Society.

Figure 2.

(A) A model for the activation of TIM by HPO_3^{2-} [3,21,22]. TIM is shown to exist mainly in an inactive open form E_o , which is converted to an active high-energy closed form E_c ($K_C \ll 1$) that is stabilized by interactions with HPO_3^{2-} . (B) A model of TIM-catalyzed deprotonation of DHAP. The cis-enediolate intermediate is stabilized by interactions with the side chains of Lys12 and His95. (C) A model for the effect of desolvation of the side chain of Glu165/167 of TIM on enzyme reactivity towards deprotonation of substrate. The difference between the observed binding energy [$(\Delta G_B)_{\text{obs}}$] and the intrinsic binding energy [$(\Delta G_B)_{\text{int}}$] is the binding energy used to drive enzyme desolvation [$(\Delta G_B)_{\text{desol}}$]. This is recovered as an enhancement in side-chain basicity toward deprotonation of substrate. (D) The structures of PGA and a *cis*-enediolate.

Figure 3.

(A) Reaction catalyzed by ketosteroid isomerase (KSI). (B) Representation of the X-ray crystal structure of KSI (PDB entry 1OH0) in a complex with equilenin, with the ligand oxygen shaded red. (C) Reaction catalyzed by diaminopimelate (DAP) epimerase. (D) Representations of X-ray crystal structures of DAP epimerase. (D1) Unliganded enzyme from *Bacillus anthracis* (PDB entry 2OTN). (D2) The covalent complex of the enzyme from *Haemophilus influenza* with LL-azi-DAP (PDB entry 2GKE). Oxygen and sulfur atoms at the active site are shaded red and yellow, respectively. (D3) A cutaway of Figure 3D2, which exposes the active site and shows the side chains for the active site bases Cys 73 and 217

Figure 4.

(A) The reaction catalyzed by designed retroaldolases. (B) The reaction catalyzed by designed Kemp eliminases. (C) Representations of HG3.17 (PDB entry 4BS0) in a complex with 6-nitrobenzotriazole. (a) A cutaway of the active site that shows the tight fit of the ligand in the catalyst active site. The ordered water molecules at the active site are shown as spheres. (b) The electron density map which shows the interaction between the bound inhibitor and the side chains of Asp127 and Gln50. Reprinted by permission from Macmillan Publishers Ltd: *Nature* (Ref 63), Copyright 2013.