Slow proton transfer from the hydrogen-labelled carboxylic acid side chain (Glu-165) of triosephosphate isomerase to imidazole buffer in D_2O

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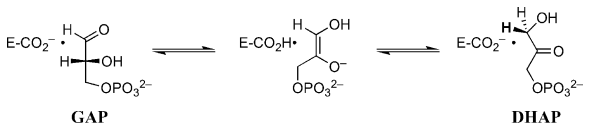
The catalytic base at the active site of triosephosphate isomerase (TIM) was labelled with –H by abstraction of a proton from substrate D-glyceraldehyde 3-phosphate to form an enzyme-bound enediol(ate) in D_2O solvent. The partitioning of this labelled enzyme between intramolecular transfer of –H to form dihydroxyacetone phosphate (DHAP), and irreversible exchange with –D from solvent was examined by determining the yields of H- and D-labelled products by ^{1}H NMR spectroscopy. The yield of hydrogen-labelled product DHAP remains constant as the concentration of the basic form of imidazole buffer is increased from 0.014 to 0.56 M. This shows that the active site of free TIM, which has an open conformation needed to allow substrate binding, adopts a closed conformation at the enediolate–complex intermediate where the catalytic side chain is sequestered from interaction with imidazole dissolved in D_2O.

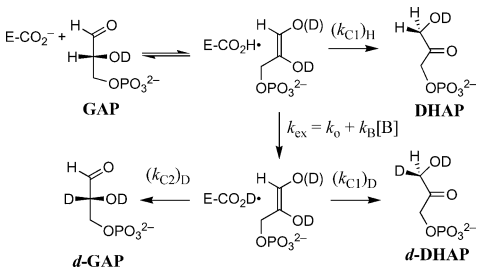
Introduction

We are interested in characterizing and understanding the mechanism for the reversible 1,2-hydrogen shift catalyzed by triosephosphate isomerase (TIM), which results in the interconversion of (*R*)-glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP, Scheme 1).[1–3] The chemical mechanism for proton transfer at the active site of TIM[4] is similar to that observed for the corresponding nonenzymatic isomerization reaction in water.[5] The carboxylate anion side chain of Glu-165† functions as a Brønsted base to abstract a proton from the α-carbonyl carbon of bound substrate,[6–11] and the developing negative charge at the carbonyl carbon is stabilized by hydrogen bonding to the neutral imidazole side chain of His-95.[8,12,13] The isomerization reaction is completed by reprotonation of the enediol(ate) intermediate at the adjacent carbon.



Scheme 1

Deprotonation of triosephosphates catalyzed by TIM is distinguished from their uncatalyzed deprotonation in water by the highly evolved network of interactions between the enzyme and the phosphate group of the substrate, which accounts for *ca.* 80% of the stabilization of the transition state for the enzymatic reaction.[1] The most striking effect of binding of phosphodianion ligands to TIM is the large *ca.* 7 Å motion of an 11-residue "mobile loop" (loop 6, Pro-166 to Ala-176) which acts as a "lid" to cover the bound ligand in the active site.[14–19] We have proposed that this loop motion has the effect of sequestering the substrate from the polar solvent water, at an active site whose operational dielectric constant is presumably smaller than for water.[2,3,20] Sequestering the substrate for TIM-catalyzed deprotonation from bulk solvent shields the basic carboxylate anion side chain of Glu-165 from reactions with bulk solvent, and favours intramolecular transfer of hydrogen from reactant to product in D_2O (Scheme 2).[2,3] The much higher fraction of C-2 hydrogen transferred from reactant to product during the TIM-catalyzed isomerization of GAP (48%) in D_2O compared with isomerization catalyzed by quinuclidinone (*ca.* 4%)[5] provides evidence that the carboxylic acid side chain of Glu-165 is shielded from interaction with solvent.[3] The nearly equal rate constants observed for partitioning of the reaction intermediate between deuterium exchange (k_{ex}, Scheme 2) and transfer to product [$(k_{C1})_H$] may reflect an active site that is partly exposed to aqueous solution. However, it is also possible that there is a very high degree of isolation of the bound substrate from solvent, and that the deuterium exchange reaction itself involves deuterium bound to basic amino acid side chains or water molecules at the enzyme active site.



Scheme 2

In this paper we consider the mechanism for the deuterium exchange reaction at the TIM–enediol(ate) complex in D_2O that converts the H-labelled E–CO_2H group to E–CO_2D (Scheme 2).

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† Our convention is to number the enzymatic residues of TIM according to the sequence for the enzyme from yeast.[7]

The first step in this reaction is effectively irreversible deprotonation of the carboxylic acid side chain of Glu-165 to form the complex of the carboxylate anion and enol(ate) followed by the fast labelling of this side chain with deuterium. Deprotonation of the carboxylic acid side chain of Glu-165 by the weakly basic solvent D_2O is expected to be relatively slow.[21] This exchange reaction will be strongly catalyzed by buffers with $pK_a \approx 7$ that are capable of freely diffusing to the enzyme active site ($k_B[B] \gg k_o$, Scheme 2) because these buffers are much more basic than D_2O.[21] If k_{ex} is for direct proton transfer between the –COOH side chain of Glu-165 and solvent, then the basic form of the buffer may catalyze this proton transfer reaction and cause an increase in k_{ex} compared with the buffer insensitive step $(k_{Cl})_H$. This would be detected as a decrease in the fraction of –H transferred from reactant GAP to product DHAP. On the other hand, if exchange involves a pool of –D at an active site that is inaccessible to buffer general bases during turnover, then the rate constant ratio $(k_{Cl})_H/k_{ex}$ will be independent of buffer concentration.

Experimental

TIM from rabbit muscle (lyophilized powder) was purchased from Sigma and had a specific activity towards isomerization of GAP of 5500–6000 units mg^{-1} at pH 7.5 and 25 °C. Glycerol 3-phosphate dehydrogenase from rabbit muscle (170 units mg^{-1}) was purchased from Boehringer. Commercially available chemicals were reagent grade or better and were used without further purification. Deuterium oxide (99.9% D) and deuterium chloride (35% w/w, 99.9% D) were purchased from Cambridge Isotope Laboratories. Sodium deuteroxide (40% wt, 99.9% D), triethanolamine hydrochloride and tetramethylammonium hydrogen sulfate were purchased from Aldrich. NADH (disodium salt), dihydroxyacetone phosphate (lithium salt), D,L-glyceraldehyde 3-phosphate diethyl acetal (barium salt), the dicyclohexylammonium salt of (*R*)-glyceraldehyde 3-phosphate diethyl acetal, and Dowex 50 W (H^+ form, 100–200 mesh, 4% cross-linked) were purchased from Sigma. Imidazole was purchased from Fluka. (*R*)-Glyceraldehyde 3-phosphate diethyl acetal was converted to GAP by following a published procedure.[3] Buffered solutions of imidazole in D_2O were prepared by dissolving neutral imidazole and NaCl in D_2O followed by addition of a measured amount of a stock solution of DCl to give the required acid–base ratio. Solution pH or pD was determined at 25 °C using an Orion Model 720A pH meter equipped with a Radiometer pHC4006-9 combination electrode that was standardized at pH 7.00 and 10.00 at 25 °C. Values of pD were obtained by adding 0.40 to the observed reading of the pH meter.

TIM was assayed by coupling the isomerization of GAP to the oxidation of NADH using glycerol 3-phosphate dehydrogenase,[3] monitored at 340 nm, by following a published procedure.[22] The concentration of GAP in these assays and in the turnover of GAP by TIM in D_2O monitored by ^1H NMR spectroscopy was obtained from the change in absorbance at 340 nm upon its complete TIM-catalyzed conversion to DHAP that was coupled to the oxidation of NADH using glycerol 3-phosphate dehydrogenase.[3]

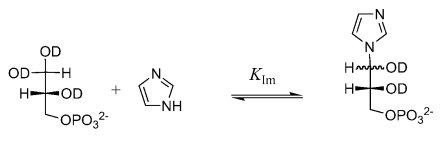
^1H NMR analyses

^1H NMR spectra at 500 MHz were recorded in D_2O at 25 °C using a Varian Unity Inova 500 spectrometer that was shimmed to give a line width of ≤0.7 Hz for each peak of the doublet due to the C-1 proton of GAP hydrate. Spectra (16–64 transients) were obtained using a sweep width of 6000 Hz, a pulse angle of 90° and an acquisition time of 7 s, with zero-filling of the data to 128 K. In order to ensure accurate integrals for the protons of interest, a relaxation delay between pulses of 127 s ($>8T_1$) was used. Baselines were subjected to a first-order drift correction before determination of integrated peak areas. Chemical shifts are reported relative to HOD at 4.67 ppm. The triplet at 0.957 ppm due the methyl group of ethanol that was present as a product of hydrolysis of GAP diethyl acetal was used as internal standard to determine the relative concentrations of substrate GAP and the products of the TIM-catalyzed isomerization reactions in D_2O.

Adducts of GAP

The ^1H NMR spectrum of 10 mM GAP in D_2O at pD 7.9 and 21 mM imidazole showed a small broad doublet at 9.59 ppm and a much larger doublet at 4.90 ppm for the carbonyl and hydrate forms of GAP.[3] The value of $f_{hyd} = 0.95$ that was determined from the spectrum obtained at 21 mM imidazole and $I = 0.15$ (NaCl) is identical to the value determined at 25 °C for GAP in D_2O at pD 7.9 maintained with triethanolamine buffer at the same ionic strength, $I = 0.15$ (NaCl).[3] These are in good agreement with $f_{hyd} = 0.96$ determined at pH 8.6 and 21 °C.[23]

No signal for the carbonyl form of GAP (11.4 mM) was observed for this compound in 0.80 M imidazole buffer at pD 7.9, but a new singlet was observed at 3.872 ppm. This very broad singlet (*ca.* 20 Hz) had an area similar to that for the doublet at 4.806 ppm due to the C-1 hydrogen of GAP hydrate. The broad line width for the signal at 3.872 ppm, along with the increase in its intensity and the corresponding decrease in the intensity of the signal for C-1 of GAP hydrate observed as the concentration of imidazole is increased, provide strong evidence that the signal is for a hydrogen of the adduct of imidazole and GAP (Im–GAP, Scheme 3). By analogy with the ^1H NMR spectrum of the corresponding imidazole adduct of glycolaldehyde, the signal at 3.872 ppm is due to the C-2 hydrogen of the Im–GAP.[20] The signal due to the C-1 hydrogen of the adduct of imidazole and GAP is not observed due to severe broadening. Other broadened new signals for the C-3 hydrogens of Im–GAP, along with a decrease in the intensity of signals for related hydrogens of GAP hydrate were observed. A complete assignment of these latter signals was not possible, because the signals for GAP hydrate and Im–GAP were not fully resolved from one another and from the quartet at 3.43 ppm due to the methylene group of ethanol that was present as a product of hydrolysis of GAP diethyl acetal. The apparent equilibrium constant K_{Im} for the formal substitution of imidazole for water at GAP hydrate to form Im–GAP in D_2O was determined by analysis of ^1H NMR spectral data of GAP [10 mM] at 25 °C in D_2O that contained 0.30, 0.50 and 0.80 M total imidazole at pD



Scheme 3

7.9 ($pK_{BD} = 7.6$, 70% free base) at $I = 0.3$ maintained with NaCl. Using eqn 1 and the observed areas for the peaks due to a single hydrogen of GAP hydrate (A_{hyd}) and Im–GAP ($A_{Im–GAP}$) at three different total imidazole concentrations ($[Im]_T$), an average value of $K_{Im} = 1.11 \pm 0.10$ was obtained. In eqn 1, A_{hyd} is the area of the doublet at 4.806 ppm due to the C-1 hydrogen of GAP hydrate and $A_{Im–GAP}$ is the area of the broad singlet at 3.872 ppm due to the C-2 hydrogen of Im–GAP.

$$K_{Im} = \frac{A_{Im–GAP}}{(A_{hyd})[Im]_T} \quad (1)$$

TIM-catalyzed isomerization of GAP in D_2O monitored by ^1H NMR

The turnover of GAP (11.4 mM, $>20 \times K_m$) by rabbit muscle TIM in a solution buffered by 0.80 M imidazole (pD 7.9) at 25 °C was monitored as described in previous work.[3] 750 μL of the reaction mixture containing buffer and substrate GAP was placed in an NMR tube and the ^1H NMR spectrum was recorded at 25 °C. This was immediately followed by the addition of TIM (*ca.* 0.03 units) in 10 μL of buffer in D_2O, and the progress of the reaction was then monitored by ^1H NMR spectroscopy at 25 °C. Each NMR spectrum was recorded over a period of 30–60 min and the reaction time t was calculated from the time at the mid-point of these analyses.

The peak areas A_{obs} for the reactants, determined by integration of ^1H NMR spectra obtained at various reaction times, were normalized according to eqn 2 to give A_P, where A_{std} and $(A_{std})_o$ are the observed peak areas of the signal due to the internal standard at time t and at $t = 0$, respectively. The disappearance of GAP was followed by monitoring the decrease in the normalized area A_{GAP} of the doublet ($J = 6$ Hz) at 4.806 ppm due to the C-1 proton of GAP hydrate, calculated using eqn 2. The fraction of GAP remaining at time t was calculated using eqn 4, where 0.53 is the fraction of GAP present as the C-1 hydrate (see above) and A_H is the normalized peak area for a *single* proton of *total* GAP at $t = 0$, calculated using eqn 3.

$$A_P = A_{obs}\left(\frac{(A_{std})_o}{A_{std}}\right) \quad (2)$$

$$A_H = (A_{GAP})_o/0.53 \quad (3)$$

$$f_{GAP} = \frac{A_{GAP}/0.53}{A_H} \quad (4)$$

$$f_{d\text{-GAP}} = \frac{A_{d\text{-GAP}}/0.53}{A_H} \quad (5)$$

The formation of *d*-GAP was followed by monitoring the appearance of the singlet at 4.805 ppm due to the C-1 proton of *d*-GAP hydrate. This singlet is shifted slightly upfield (0.001 ppm) from the midpoint of the doublet at 4.806 ppm due to the C-1 proton of GAP hydrate, as a result of the presence of deuterium at C-2. The fraction of GAP converted to *d*-GAP was calculated using eqn 5, where $A_{d\text{-GAP}}$ is the normalized area of the singlet due to the C-1 proton of *d*-GAP hydrate calculated using eqn 2, and 0.53 is the fraction of *d*-GAP present as the C-1 hydrate.

The formation of DHAP was followed by monitoring the appearance of the singlets at 4.284 ppm and 3.358 ppm due to the two protons of the CH_2OD groups of the keto and hydrate forms of DHAP, respectively. The formation of *d*-DHAP was followed by monitoring the appearance of the triplet ($J_{HD} = 2$–3 Hz) at 4.257 ppm and the broad singlet at 3.339 ppm due to the single proton of the CHDOD groups of the keto and hydrate forms of *d*-DHAP, respectively. The yields of DHAP and *d*-DHAP from the TIM-catalyzed reaction of GAP were calculated using equations analogous to eqn 4 and 5, as described in earlier work.[3]

Results

The products of the TIM-catalyzed reactions of GAP in D_2O at pD 7.9 ($I = 0.15$, NaCl) in solutions buffered by 0.021 and 0.083 M imidazole were determined by ^1H NMR analysis in earlier work.[3] The same protocol was followed to determine the yields of the products of the TIM-catalyzed reactions of GAP in D_2O at pD 7.9 and in the presence of 0.80 M imidazole buffer ($pK_{BD} = 7.6$, 70% free base) at $I = 0.3$ (NaCl). ^1H NMR analysis of GAP under the conditions used for this TIM-catalyzed reaction showed that nearly 50% of the GAP hydrate was converted to Im–GAP (Scheme 3) illustrating that 1.0 M imidazole and D_2O solvent exhibit a similar affinity for addition to GAP. The interconversion of GAP and GAP hydrate is slow on the NMR time scale, but much faster than our ^1H-NMR analyses of product yields.[23] The observation of broad signals for the different hydrogens of Im–GAP, and of the collapse of the minor peak at 9.59 ppm for the C-1 hydrogen of the carbonyl form of GAP, provide evidence that interconversion of these compounds is faster than the interconversion of GAP and GAP hydrate.

The yields of the products of the reaction of GAP in D_2O at pD 7.9 that contains 0.80 M imidazole and 0.042 units mL^{-1} of TIM were determined by ^1H NMR analysis. A full description of these analyses is given in an earlier study of the TIM-catalyzed reactions at pD 7.9 in solutions buffered by 0.021 and 0.083 M imidazole.[3] Fig. 1 (▼, 0.80 M imidazole buffer) shows the change with time in the fractional yield of DHAP [$(f_{DHAP})_E$] from the

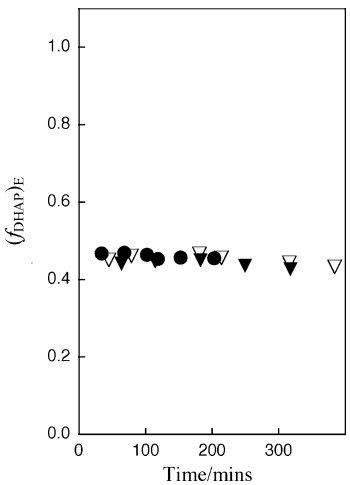
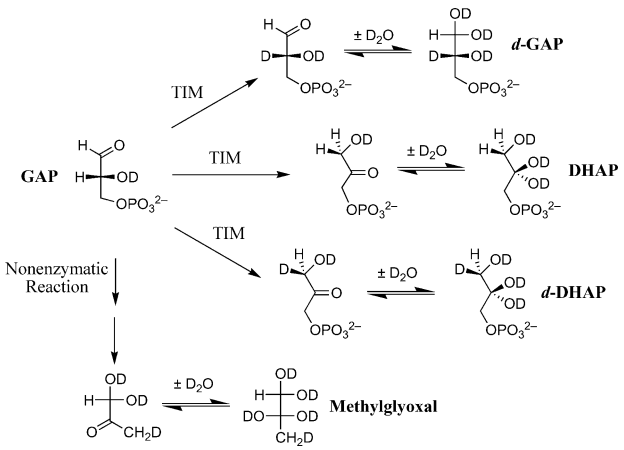


Fig. 1 The change with time in the fractional yields of DHAP from the TIM-catalyzed reaction of GAP in D_2O at 25 °C and in the presence of increasing concentrations of imidazole buffer (pD 7.9), calculated from the yields of all of the products of the enzyme-catalyzed reaction using eqn 6. Key: (▼), reaction in the presence of 0.80 M imidazole buffer; (▽), reaction in the presence of 0.083 M imidazole buffer; (●), reaction in the presence of 0.021 M imidazole buffer.



Scheme 4

TIM-catalyzed reactions of GAP calculated from eqn 6, where, $f_{d\text{-GAP}}$, f_{DHAP} and $f_{d\text{-DHAP}}$ are the total yields of products (Scheme 4). The values of $(f_{\text{DHAP}})_{\text{E}}$ are greater than f_{DHAP} determined from equations similar to 4 and 5, because of the 25% yield of methylglyoxal from the nonenzymatic elimination reaction of GAP (Scheme 4).[5]

$$(f_{\text{DHAP}})_{\text{E}} = \frac{f_{\text{DHAP}}}{f_{d\text{-GAP}} + f_{\text{DHAP}} + f_{d\text{-DHAP}}} \qquad (6)$$

The small decrease in $(f_{\text{DHAP}})_{\text{E}}$ with time is due to the slow TIM-catalyzed exchange of the C-1 hydrogens of DHAP for deuterium from solvent D_2O. The initial product yield $(f_{\text{DHAP}})_{\text{E}} = 0.45 \pm 0.01$ was determined by making a short extrapolation of these data from Fig. 1 to $t = 0$. A similar procedure was followed in determining values of $(f_{d\text{-DHAP}})_{\text{E}} = 0.37 \pm 0.01$ and $(f_{d\text{-GAP}})_{\text{E}} = 0.18 \pm 0.01$ for the initial yields of deuterium-labelled products of the rabbit muscle TIM-catalyzed reactions of GAP at pD 7.9 (0.80 M imidazole buffer). Fig. 1 also shows data for the enzyme-catalyzed isomerization reaction in the presence of 0.021 (●) and 0.083 M (▽) imidazole buffer reported in earlier work from which limiting values $(f_{\text{DHAP}})_{\text{E}} = 0.48$ and $(f_{\text{DHAP}})_{\text{E}} = 0.49$ were determined by extrapolation of the experimental data from Fig. 1 to zero time.[3]

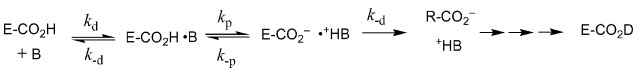
Discussion

We reported earlier that identical yields of DHAP, *d*-DHAP and *d*-GAP were obtained from the TIM-catalyzed reactions of GAP in D_2O at pH 7.5 (0.048 M triethanolamine buffer) and at pD 7.9 (0.021 and 0.083 M imidazole buffer). We have now examined the TIM-catalyzed reactions of GAP at pD 7.9 buffered with 0.80 M imidazole in order to determine whether there is any detectable deprotonation of ^{1}H-labelled enzyme by this buffer base that causes a decrease in the yield of DHAP from intramolecular transfer of ^{1}H from reactant to product in D_2O (Scheme 2). The fractional yield of DHAP from the TIM-catalyzed reaction of GAP in the presence of 0.80 M imidazole buffer $[(f_{\text{DHAP}})_{\text{E}} = 0.45]$ is marginally lower than the range of yields $[(f_{\text{DHAP}})_{\text{E}} = 0.47\text{–}0.49]$ reported in three earlier experiments at lower concentrations of imidazole (0.083 M and 0.021 M) or triethanolamine (0.048 M) buffers at pD 7.9. We conclude that the imidazole buffer effect on product yields is either very small or negligible. In the experiments at lower imidazole concentrations, a rate constant ratio of $(k_{\text{Cl}})_{\text{H}}/k_0 = 0.923$ for partitioning of the intermediate between transfer of –H to product DHAP and to solvent water can be calculated based on the observation of an average value of 48% intramolecular ^{1}H transfer from reactant GAP to product DHAP in D_2O.[3] At the higher concentration of imidazole (0.8 M), the observation of 45% intramolecular transfer product permits an estimate of the rate constant ratio $[(k_{\text{Cl}})_{\text{H}}/(k_0 + k_{\text{B}}[\text{B}])] = 0.818$ for partitioning of the intermediate between transfer of –H to product DHAP, and to both solvent water *and* the basic form of imidazole buffer. Thus the apparent *ca.* 3% decrease in the yield of DHAP for the reaction in the presence of 0.80 M imidazole buffer is consistent with a rate constant ratio of $(k_{\text{Cl}})_{\text{H}}/k_{\text{B}}[\text{B}] = 7.2$ for partitioning of the intermediate between transfer of –H to product DHAP and to the basic form of imidazole buffer (0.56 M). The *ca.* 3% decrease in the yield of DHAP might also represent experimental error or a small medium effect on product yields.

Buffer effects on deprotonation of acidic groups at protein catalysts have been observed. Carbonic anhydrase and superoxide dismutase catalyze the already fast hydration of CO_2 and the dismutation of superoxide to HOOH and H_2O, respectively. These enzymes are so effective at reducing the already low barriers to the chemical steps of the catalyzed reaction, that proton transfer between solvent and the protein becomes rate determining for reactions in unbuffered solutions.[24] In the case of carbonic anhydrase, the addition of buffer bases causes k_{cat} to increase from *ca.* 10^4 s^{-1} for rate determining proton transfer from E–Zn^{2+}–OH_2 to water to generate nucleophilic Zn^{2+}–OH^-, to *ca.* 10^6 s^{-1} for rate determining addition of zinc-bound hydroxide E–Zn^{2+}–OH^- to CO_2 to form Zn^{2+}–OCO_2^{2-}. The midpoint for this change in rate determining step is observed at *ca.* $[\text{B}] = 10^{-3}$ M.[25–28] The value of k_{cat} becomes buffer independent at $[\text{B}] \gg 10^{-3}$ M.

The Brønsted plot of second-order rate constants for deprotonation of E–Zn^{2+}–OH_2 (human carbonic anhydrase II) shows a downward break at pH 7.6, where the pK_a for the enzyme-bound Zn^{2+}–OH_2 is equal to the pK_a of the base catalyst,[28] and a limiting second-order rate constant of $k_{\text{B}} = 10^9$ M^{-1} s^{-1} for thermodynamically favorable proton transfer from the enzyme to external buffer bases. There is good evidence that proton transfer from E–Zn^{2+}–OH_2 to the external base occurs through intervening water molecules that connect the enzyme active site to external solvent.[26]

Strong buffer catalysis for exchange of deuterium at an exposed active site residue is predicted by the Eigen mechanism for proton transfer between electronegative atoms (Scheme 5), in which there is only a small intrinsic barrier for the chemical proton transfer step ($k_{\text{p}} \approx k_{-\text{p}} \approx 10^{10}$ s^{-1}).[21] The rate constant for buffer deprotonation of such an acid, E–CO_2H (Scheme 5), is equal to k_{d} for diffusion-controlled formation of the reactive complex between acid and base ($k_{\text{B}} = k_{\text{d}} = 5 \times 10^9$ M^{-1} s^{-1}, Scheme 5), when proton transfer is thermodynamically favorable. Thermodynamically uphill proton transfer occurs by equilibrium transfer to give the unstable product complex, followed by rate determining separation of this complex



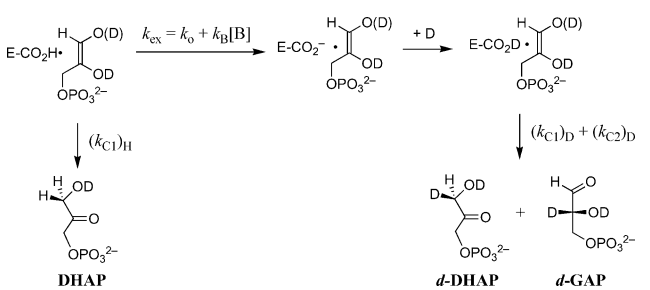
Scheme 5

(k_{-d}). In this case, the observed rate constant k_B for proton transfer to a buffer base is given by eqn 7. Water is a weak base ($pK_a = -1.74$ for the conjugate acid hydronium ion) but is present in a large concentration of 55.5 M. The value of $k_w = 5 \times 10^2$ s^{-1} calculated using eqn 7 for deprotonation of an acid of pK_a 7 by water is much smaller that the pseudo-first order rate constants for the near diffusion-limited deprotonation of this acid by $\geq 10^{-3}$ M imidazole base ($pK_{BD} = 7.6$), and buffer catalysis of these proton transfer reactions is therefore observed at neutral pH.[21]

$$(k_B = k_{-d}[(k_d/k_{-d})(k_p/k_{-p})] = (k_d)(k_p/k_{-p}) = (k_d)[(K_a)_{RCOOH}/(K_a)_{BH+}]) \quad (7)$$

Our results show the exchange between the protonated carboxylic side chain of Glu-165 and deuterium from D_2O is, at best, barely promoted by the addition of base concentrations of up to 0.56 M, so that $k_o > k_{ex}[B]$ ([B] = [imidazole] = 0.56 M) for the exchange reaction shown in Scheme 2. We conclude that the carboxylic acid side chain of Glu-165 is strongly shielded from solvent, in comparison to E–Zn^{2+}–OH_2 at carbonic anhydrase, whose deprotonation is strongly promoted by buffer catalysts. Free TIM that is not bound to substrate has an open active site that is presumably accessible to deprotonation by imidazole.[29,30] If so, then movement of flexible loop 6 of TIM,[18,31–34] and other conformational changes that occur upon substrate binding and conversion to the enediol(ate) reaction intermediate must shield the active site from interaction with small solutes in bulk solvent. This is consistent with a large body of recently summarized experimental evidence that the active site of TIM after binding of substrate is strongly shielded from interaction with bulk solvent.[2,3]

A lower limit for the kinetic effect of the shielding of the active site can be estimated as follows. From the pH-dependence of the rate of inactivation of yeast TIM by covalent inhibitor chloroacetol sulfate, the pK_a of the carboxylic acid side chain of Glu-165 in free TIM is predicted to be 3.9.[35] The pH dependence of binding of the enediolate phosphate analogue, phosphoglycolic acid, to rabbit muscle TIM is consistent with the titration of a group with an apparent pK_a of 7.5, which has been assigned to the carboxylate chain of Glu-165 in the TIM–enediolate phosphate complex.[36] The upper limit for k_o for deprotonation of a solvent accessible carboxylic acid side chain of Glu-165 (E–COOH, Scheme 6) by water lies in the range $\approx 10^5$–100 s^{-1} for deprotonation of a side chain of pK_a 4–7.5. The minimal deprotonation of the carboxylic acid side chain of Glu-165 by imidazole shows that k_B[0.56 M] is at least 10-fold smaller than k_o and establishes an upper limit of $\approx 10^4$ M^{-1} s^{-1} for k_B. By comparison, a value of $k_B \approx 10^9$ M^{-1} s^{-1} is expected for deprotonation of fully solvent accessible E–COOH by imidazole ($pK_{BD} \approx 7.6$ in D_2O). We conclude that deprotonation



Scheme 6

of this side chain by imidazole is at least 10^6 fold slower than deprotonation of a solvent accessible carboxylic acid of the same pK_a.

Aldose–ketose isomerization with intramolecular transfer of hydrogen in D_2O follows a type of conducted-tour reaction mechanism first described by Cram *et al.*[37,38] for allylic rearrangement reactions in organic solvents, since in both cases intramolecular proton transfer occurs at a complex that rearranges and collapses to form products faster than it reacts with bulk solvent. We suggest that these exchange reactions are coupled to intramolecular transfer of deuterium from O-2 to O-1 of the enediol(ate) intermediate. This is shown in Scheme 7, a working model for the reaction of hydrogen labelled reactant in D_2O that is consistent with known relevant data. Three main pathways for isomerization are shown in Scheme 7:

Pathway A

Isomerization where hydron transfer between the two enediolate oxygens occurs by consecutive proton transfer reactions of Glu-165. This leads to the formation of deuterium-labelled product and not to incorporation of deuterium into unreacted substrate. The observation that a H95N mutant of TIM catalyzes transfer of tritium label from solvent to product but not to unreacted substrate, when the substrate is either GAP or DHAP,[12] provides evidence that A (the *criss-cross* mechanism) is the dominant reaction pathway when there is no stabilization of the enediolate intermediate by hydrogen bonding to His-95. The reactions catalyzed by wildtype TIM in solvent D_2O result in the faster transfer of deuterium from solvent D_2O into product than to unreacted substrate when the substrate is *either* DHAP *or* GAP.[2,3] This provides evidence that pathway A is also significant even when the basicity of the enolate oxyanion is reduced by hydrogen bonding to His-95.

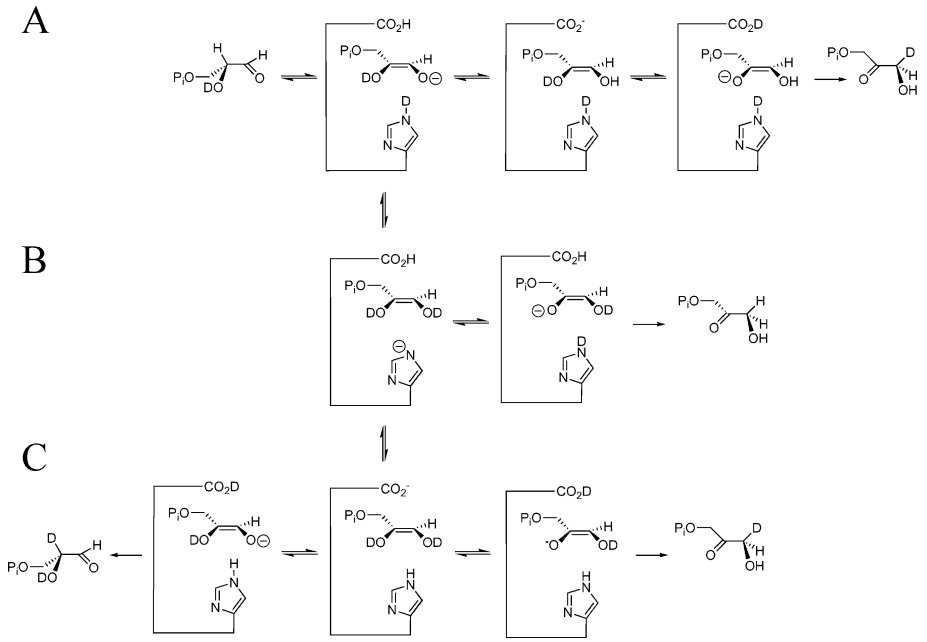
Pathway B

Isomerization with transfer of deuterium from O-2 to O-1 of the enediol(ate) intermediate that is promoted by His-95. This may be the major pathway for isomerization with intramolecular transfer of hydrogen label from reactant to product.

Pathway C

Proton exchange between Glu-165 and His-95 through the enediol(ate) intermediate. The Glu-165 labelled with deuterium that forms by this pathway may react with the intermediate to give either deuterium-labelled reactant or product. Our data neither confirm nor exclude the proposal (not shown in Scheme 7) that there is also hydron exchange between substrate and D_2O sequestered at the enzyme active site (not shown in Scheme 7).[3,39]

Pathway C shows transfer of a solvent-derived hydron from His-95 to product. Irwin Rose *et al.* have reported that labelling of TIM with tritium from water in a rapid pulse, followed by fast dilution into unlabelled water that contains DHAP leads to the transfer of one mole of tritium from solvent to the product of isomerization in the chase solution.[40] This may reflect labelling of His-95 in the tritium pulse, followed by transfer of label to the product by pathway C in the *chase* experiment.



Scheme 7

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