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Difference FTIR Studies of Substrate Distribution in Triosephosphate Isomerase

Hua Deng^{*,†}, Jayson Vedad, Ph.D.[‡], Ruel Z. B. Desamero, Ph.D.[‡], and Robert Callender[†]

[†]Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461, United States

[‡]Programs in Chemistry and Biochemistry, CUNY Graduate Center and Department of Chemistry, York College of CUNY, Jamaica, New York 11451, United States

Abstract

Triosephosphate isomerase (TIM) catalyzes the interconversion between dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP), via an enediol(ate) intermediate. Determination of substrate population distribution in the TIM/substrate reaction mixture at equilibrium and characterization of the substrate-enzyme interactions in the Michaelis complex are ongoing efforts toward the understanding of the TIM reaction mechanism. By using isotope-edited difference Fourier transform infrared studies with unlabeled and ¹³C-labeled substrates at specific carbon(s), we are able to show that in the reaction mixture at equilibrium the keto DHAP is the dominant species and the populations of aldehyde GAP and enediol(ate) are very low, consistent with the results from previous X-ray structural and ¹³C NMR studies. Furthermore, within the DHAP side of the Michaelis complex, there is a set of conformational substates that can be characterized by the different C2=O stretch frequencies. The C2=O frequency differences reflect the different degree of the C2=O bond polarization due to hydrogen bonding from active site residues. The C2=O bond polarization has been considered as an important component for substrate activation within the Michaelis complex. We have found that in the enzyme-substrate reaction mixture with TIM from different organisms the number of substates and their population distribution within the DHAP side of the Michaelis complex may be different. These discoveries provide a rare opportunity to probe the interconversion dynamics of these DHAP substates and form the bases for the future studies to determine if the TIM-catalyzed reaction follows a simple linear reaction pathway, as previously believed, or follows parallel reaction pathways, as suggested in another enzyme system that also shows a set of substates in the Michaelis complex.

Graphical Abstract

*Corresponding Author: hdeng12@einstein.yu.edu.

ORCID

Hua Deng: 0000-0002-9503-7572

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Triosephosphate isomerase (TIM) plays an important role in the glycolic pathway and is essential for efficient energy production. Malfunction of TIM in humans leads to hemolytic anemia, neurologic dysfunction, and often early death.¹ TIM is one of the most intensively studied enzymes since 1970s; a simple search in the Protein Databank revealed more than 200 structures. Recently, there is renewed interest in this enzyme due to the discovery of its association with Alzheimer's disease² and the discovery of allosteric inhibitors specific for TIM from parasite *Trypanosoma cruzi* (TcTIM), the causative agent of Chagas' disease.^{3–10}

TIM catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP) by an enolization mechanism involving a cis-enediol intermediate (Scheme 1). In the forward direction, active site His95 forms a hydrogen bond to the DHAP C2=O bond. This hydrogen bond polarizes C2=O to facilitate proton abstraction from the DHAP C1 carbon by the active site Glu165 residue to form the enediol(ate) reaction intermediate, with possible concomitant proton transfer from His95 to the C2=O oxygen. In the next step, a proton transfers from Glu165 to the C2 carbon and from C1-OH to His95 for the product GAP to complete the reaction.

In a classical enzyme kinetic study using deuterium/tritium isotopes, a free energy profile along the reaction pathway for the TIM-catalyzed reaction was determined.¹¹ On the basis of the parameters derived from this energy profile, it has been estimated that DHAP comprises ~65% of the enzyme-bound species while significant amounts of enediol intermediate (~20%) and GAP (~15%) are also present in the TIM/ substrate reaction mixture at equilibrium.¹² Several studies using X-ray crystallography and spectroscopic

methods have been conducted to determine substrate population distributions in the TIM/ substrate reaction mixture directly. X-ray structural studies on TIM cocrystallized with the substrate suggested that the most likely molecular species bound in the active site is DHAP, consistent with the estimated DHAP population based on the free energy profile. However, the existence of the enediol(ate) intermediate or GAP in the TIM/substrate crystal could not be verified.¹³ Early ³¹P NMR studies also suggested that the dominant species was keto DHAP and the percentage of aldehyde GAP was low.¹⁴ In a ¹³C solid-state and solution NMR study on the TIM/substrate reaction mixture at equilibrium (with TIM/substrate concentration ratio of ~2:1 to ensure most substrates were bound to TIM), it was found that ~95% of the bound substrate was keto DHAP, whereas ~ 5% of the bound substrate was hydrated GAP, in which RHC1=O becomes RHC1(OH)₂. Interestingly, no enediol-(ate) intermediate or aldehyde GAP could be detected in the TIM reaction mixture by ¹³C NMR.¹² The ¹³C resonance of DHAP is quite broad, indicating a fast exchange among various forms of DHAP bound in TIM and free in solution in the reaction mixture. The exact forms of these DHAP substates were not determined by the NMR studies.

Early Fourier transform infrared (FTIR) studies of the TIM/ substrate reaction mixture at equilibrium showed two C2=O stretch bands from bound DHAP.¹⁵ The major lower-frequency band was assigned to the bound DHAP with polarized C2=O bond by active site interactions, interpreted as the “active form” of DHAP. The minor higher-frequency band was assigned to the bound DHAP with unpolarized C2=O bond, interpreted as the “inactive form” of DHAP. On the basis of the FTIR results, it was suggested that DHAP binds to TIM to form the inactive substate first and then proceeds to the activated substate before the chemical step in a linear fashion.¹⁵ Apparently, these DHAP substates are in fast exchange in the NMR time scale so that only one resonance was observed in the NMR studies.

Although TIM-bound GAP has been detected by FTIR in several TIM mutant/substrate reaction mixtures at equilibrium,^{16,17} GAP was not identified in the reaction mixture using wild-type TIM.^{15–17} One possibility for the lack of FTIR evidence for GAP in wide-type TIM was that the frequency of the C1=O stretch modes of the TIM-bound GAP was below 1700 cm⁻¹ so that the intense protein absorbance prevented reliable determination of such modes by normal FTIR difference methods between the TIM/substrate binary complex and TIM, as demonstrated in several studies (also see Figure 2 in Results).^{15–17}

In the current study, we have developed simple enzymatic methods to form ¹³C-labeled DHAP at specific carbons. The isotope-edited difference FTIR method was applied to the TIM/substrate reaction mixture at equilibrium. In this method, a difference spectrum is formed between two protein-ligand complexes, one of which is specifically labeled with a stable isotope. Vibrational modes that are associated with the isotopic tag show as spectral shifts in the difference spectrum, whereas all other bands from either the protein or ligand will be canceled. When the concentrations of the labeled and unlabeled ligands are carefully matched, this difference method can virtually eliminate the protein interference in the difference spectrum, as shown in our previous studies on various enzyme systems (for a recent review, see ref 18). Thus, the observation of IR bands from the reaction intermediate and/or GAP is expected using this method if these species exist in significant amount in the TIM/substrate reaction mixture.

Our results from the isotope-edited difference FTIR measurements show that it is possible to determine the substrate population distribution in the TIM/substrate reaction mixture at equilibrium quantitatively. In addition, the population distribution of the substates with different C2=O bond polarizations within the DHAP side of the Michaelis complex can also be determined. Apparently, the different substrate C=O bond polarizations in these substates of the Michaelis complex indicate different interaction energies to the C O bond. The observation of all of these substates in our studies show that their overall energies are similar, thus the differences in the local interaction energies to the C=O bond must be compensated by the interactions to the other part of the substrate, especially the phosphodianion moiety, as elegantly revealed by Richard's lab.^{19,20} The implications of these findings on the TIM reaction mechanism will be discussed.

MATERIAL AND METHODS

TIM from baker's yeast (YeTIM), DHAP, DL-GAP, adenosine 5'-triphosphate (ATP), glycerokinase, oxidase, and catalase were purchased from Sigma. 2-¹³C, 1,3-¹³C, and uniformly ¹³C-labeled glycerols were purchased from Cambridge Isotope Laboratories. For FTIR studies, the TIM protein samples were first dialyzed against 50 mM tris buffer at pH 7.8 with 50 mM NaCl and then washed with D₂O buffer four times (each with 40-fold dilution) in a Centricon concentrator. The concentrations of the TIM sample were determined by UV-vis spectroscopy using molar extinction coefficient $\epsilon_{280} = 26.9 \text{ cm}^{-1} \text{ mM}^{-1}$. The pH value is reported without deuterium correction on the measured pH values. An appropriate amount of concentrated DHAP stock solution, typically 150 mM at pH 7 in D₂O, was then added to the TIM sample to form the TIM/DHAP reaction mixture, typically at 10 mN:10 mM for FTIR measurements. The unit mN is referring to the concentration of the active site (subunit) to reflect the fact that TIMs exist as dimmers in solution.

The TcTIM gene was synthesized and subcloned to the pJ414 plasmid by DNA2.0. A DNA sequence to code a six-residue HIS tag was added to the 5' end of the gene, followed by the DNA sequence to code the amino acid sequence ENLYFQS to create the cleavage point for Tobacco Etch Virus (TEV) Protease. The entire DNA sequence added to the 5' end of the TcTIM gene was ATGGGTAGCTCTCATCATCAC-CATCATCACAGCAGCGGCGAGAACCTGTATTTTCA-GAGC (corresponding to the amino acid sequence MGSSHHHHHHSSGENLYFQS). The pJ414 plasmid with the TcTIM gene was transformed into the *Escherichia coli* BL21(DE3) cell. Protein expression was conducted by our published procedure.²¹ At the end of the culture, the cells are collected, resuspended in 50 mM tris buffer, and lysed by ultrasound. The cell debris was removed by centrifugation, and the supernatant was loaded onto a 5 mL Ni⁺ column equilibrated in 50 mM, pH 7.8 tris buffer with 0.5 M NaCl. After washing the column by 50 mM, pH 7.8 tris buffer with 0.5 M NaCl/20 mM imidazole, TcTIM with the His tag was eluted by 0.5 M NaCl/200 mM imidazole from the Ni⁺ column. TEV with a His tag was mixed with eluted TcTIM (1:50 ratio) in a dialysis bag and dialyzed for 2 h at room temperature and then overnight at 4 °C. TEV cleaves the HIS tag on TcTIM with most of other residues except a single Ser residue before the native initial Met residue. The TEV reaction mixture was then passed through a Ni⁺ column to remove HIS tags and TEV. The purity of thus-purified TcTIM was checked with sodium dodecyl sulfate gel and was typically >90% pure. The

typical yield of the TcTIM protein by this procedure is $>50 \text{ mg L}^{-1}$ culture. The TcTIM sample for FTIR studies was similarly prepared as YeTIM. The concentrations of the TcTIM sample were determined by UV-vis spectroscopy using molar extinction coefficient $\epsilon_{280} = 27.3 \text{ cm}^{-1} \text{ mM}^{-1}$.

^{13}C -labeled DHAPs were prepared from correspondingly labeled glycerols. Glycerol (25 mg) was mixed with 220 mg of ATP, 200 μL of 1 M MgCl_2 , and 50 units of glycerokinase in 5 mL of H_2O to convert glycerol to L-glycerol 3-phosphate (G3P). The pH of the reaction mixture was maintained between 7 and 7.5 at room temperature by titration of NaOH. After the completion of the reaction, as determined by NMR, the reaction mixture was washed by charcoal to remove ATP and ADP. The solution was then concentrated to 2 mL and passed through a Dowex 50W column (H form, 1 cm $\times$ 5 cm) to remove Mg^{2+} . The fractions with L-G3P were collected and titrated to pH 7.5 using 1N LiOH. An aliquot of 1–1.5 mL of G3P (10–15 mg, Li^+ salt) was loaded into a NMR tube. Prewashed 20 units of G3P oxidase and 20 units of catalase were then added to the NMR tube. Oxygen gas was bubbled into the reaction mixture solution at room temperature to convert L-G3P into DHAP. The reaction typically completed within 20 min, as monitored by NMR, and the side products, such as phosphate and methylglyoxal, were less than 2%. The reaction mixture solution was filtered to remove proteins and then lyophilized and stored at -80°C until ready to use.

FTIR spectroscopy was performed on a Magna 760 Fourier transform spectrometer (Nicolet Instrument Corp., WI) using a MCT detector. We used a two-position sample shuttle to alternate between the unlabeled sample and labeled sample positions; this procedure substantially decreased the spectral contribution of residual water vapor after subtraction. Both TIM/DHAP and TIM/ ^{13}C -labeled DHAP sample solutions were simultaneously loaded into a dual cell shuttle accessory. CaF_2 windows with 15 μm Teflon spacers were used. The typical sample volume was 5 μL . Spectra were recorded in the range of 1100–4000 cm^{-1} with 2 cm^{-1} resolution. A Blackman-Harris three-term apodization and a Happ-Genzel apodization were applied, respectively. Omnic 7.1 (Nicolet Instruments, Corp.) software was used for data collection and analysis.

RESULTS

It has been shown that the substrate-enzyme interactions at the active site may be characterized quantitatively by vibrational spectroscopy in terms of interaction energies, chemical bond length changes, and distortions of molecular groups (cf. refs22–24). For a bound substrate in TIM, our FTIR studies will be focused on the $\text{C}=\text{O}$ stretch mode of DHAP/GAP. In general, the $\text{C}=\text{O}$ stretch frequency is sensitive to electrostatic interactions, including ionic interactions with external ions, hydrogen bonding interactions, and polar dipole-dipole interactions, on the $\text{C}=\text{O}$ bond. There have been two general approaches to describing the electrostatic interactions on the $\text{C}=\text{O}$ bond quantitatively in terms of $\text{C}=\text{O}$ stretch frequencies. One is the so-called Badger and Bauer rule, which states that the enthalpy of formation of a hydrogen bond is related linearly to the vibrational frequency shift of the molecular group.²⁵ The relationship between the $\text{C}=\text{O}$ stretch frequency and the interaction energy of a simple $\text{C}=\text{O}$ -containing molecule interacting with various cations and water has been investigated by ab initio calculations, and it was found that the shift in

the C=O stretching frequency fits a linear correlation with the computed interaction energy in a wide range of values up to ~25 kcal/mol. The results showed that ~2 cm⁻¹ shift of the C=O stretch frequency correlates to 1 kcal/mol interaction energy change.²⁶ These computational results agree remarkably well with the experimentally determined correlation between the C=O stretch frequency and enthalpy of hydrogen bond formation on similar simple ketones observed in a smaller energy range (<10 kcal/mol).²⁷ For more complex molecules, such as a molecule that contains an amide, the C=O stretch frequency can still be used to estimate the interaction energy at the active site of an enzyme but will require the X-ray structure and computational assistance.²⁸

The second approach is to relate the frequency shift of a molecular group quantitatively to the electric field it experiences. Recently, it was found that the C=O stretch frequency of a molecule is linearly correlated to the electric field near the C=O bond, as determined by Stark spectroscopy. This method may determine the electric field near the C=O bond of the protein-bound molecule without the knowledge on the active site structure.²⁹

In addition to probing the active site interaction energies or electrostatic environments, the substrate C=O stretch frequency shift upon substrate binding to an enzyme can often be used to predict the enzyme activity. It has been found that the C=O stretch frequency change is linearly correlated to the activity (k_{cat}) of the enzyme variants (mutants) in a number of enzyme systems.²⁹⁻³¹ However, no such correlation seems to exist, as demonstrated in a number of FTIR studies on TIM and its mutants.^{15,17,32-34}

Here, the substrate C=O stretch frequencies in solution will be determined first and then the C=O frequencies and intensities of the bound substrates will be determined to characterize the active site interactions and the substrate population distribution in the enzyme. Finally, the differences of the substrate binding in two isozymes, YeTIM and TcTIM, will be studied.

FTIR Studies of GAP and DHAP in Solution

Our FTIR studies will be focused on the C=O/C=C stretch region (1600–1750 cm⁻¹). It has been shown by ³¹P NMR spectroscopy that most of GAP exists in the hydrated form in solution, where C1=O is hydrated to form C1(OH)₂ diol (not observed in our FTIR spectra).^{14,35} The aldehyde form of GAP also exists in small amount, as shown by a single low-intensity resonance in the NMR spectrum.³⁵ The FTIR spectrum of GAP shows two C=O stretch bands at 1730 and 1745 cm⁻¹ (see Figure 1a), suggesting at least two conformations for the aldehyde form of GAP. These two aldehyde GAP conformations interconvert fast in the NMR time scale but slowly in the vibrational time scale.

For DHAP, keto and hydrated forms exist in comparable amounts in solution and they interconvert in the subsecond time scale.^{14,36} Like GAP, only a single resonance was observed for the keto form of DHAP in solution NMR.^{12,14,37} In FTIR studies, two DHAP C2=O stretch bands at 1728 and 1742 cm⁻¹ are observed with intensity ratio of ~2:1 (Figure 1b). These bands shift down by 43 cm⁻¹ to 1685 and 1699 cm⁻¹, respectively, upon 2-¹³C labeling (Figure 1c); thus, both can be assigned to the DHAP C2=O stretch mode. The results show that keto DHAP exists in two conformations in solution and they interconvert fast in the NMR time scale but slowly in the vibrational time scale. On the

basis of the frequency-interaction energy and frequency-bond length correlations derived for similar carbonyl compounds, the 14 cm^{-1} frequency difference between the $\text{C}=\text{O}$ stretches of the two DHAP conformations suggests an $\sim 7\text{ kcal/mol}$ stronger hydrogen bonding and $\sim 0.007\text{ \AA}$ longer bond length for the DHAP conformation with 1728 cm^{-1} $\text{C}=\text{O}$ stretch frequency.^{22–24}

To investigate the structural features of the two keto forms of DHAP in solution, vibrational normal mode calculations using ab initio methods were performed. These calculations were performed using the b3lyp/6-31g* functional on the isolated DHAP molecule with various starting conformations. After geometry optimization, two stable structures were obtained. In one structure, the $\text{C}=\text{O}$ and C1-OH bonds were on the same side of the C1-C2 bond (in syn conformation) to form an intramolecular hydrogen bond, $\text{C1-OH}\cdots\text{O}=\text{C2}$ (distance $\sim 2\text{ \AA}$ and with the $\text{O-H}\cdots\text{O}$ angle of $\sim 90^\circ$). The calculated $\text{C}=\text{O}$ stretch frequency was 1710 cm^{-1} . In the other optimized stable DHAP structure, the $\text{C}=\text{O}$ and C1-OH bonds were not on the same side of the C1-C2 bond, so the $\text{C}=\text{O}$ bond was isolated. The calculated $\text{C}=\text{O}$ stretch frequency in this DHAP conformation was 1742 cm^{-1} , 32 cm^{-1} higher than that in the syn conformation. Although such preliminary calculations did not reproduce the exact match to the experimentally observed frequency differences, they did suggest that the formation/breaking of the internal hydrogen bond is the reason for the two observed $\text{C}=\text{O}$ stretch bands in the keto DHAP and aldehyde GAP in solution.

FTIR Difference Studies of the TIM/Substrate Reaction Mixture at Equilibrium

The TIM/substrate reaction mixture at equilibrium may be formed by adding a stoichiometric amount of DHAP to the concentrated TIM solution. In a pioneer study, Belasco and Knowles demonstrated that it was possible to probe the interaction between TIM and its substrate DHAP in the reaction mixture by a difference FTIR method. In this method, the $\text{C}=\text{O}$ stretch bands of bound DHAP in the TIM/DHAP mixture were revealed by subtracting the TIM IR spectrum from the TIM/DHAP IR spectrum. A major $\text{C}=\text{O}$ stretch band from TIM-bound DHAP was found at 1714 cm^{-1} , red-shifted by about 20 cm^{-1} relative to the average solution DHAP $\text{C}=\text{O}$ stretch band and was suggested to be the active form of the bound substrate due to its more polarized $\text{C}=\text{O}$ bond. Another minor band was found at 1732 cm^{-1} , relatively unchanged from the average of the DHAP solution $\text{C}=\text{O}$ bands and was suggested to be an inactive form of the bound DHAP due to its minimum $\text{C}=\text{O}$ polarization.¹⁵ The assignments of these two $\text{C}=\text{O}$ bands were confirmed by their disappearance when the measurements were performed on the reaction mixture samples in D_2^{18}O , in which the oxygen of DHAP $\text{C}=\text{O}$ was exchanged with ^{18}O to cause red shifts of the $\text{C}=\text{O}$ modes.¹⁵ The application of this difference method on the TIM/DHAP mixture and TIM is demonstrated in Figure 2.

Figure 2a,b shows the FTIR spectrum of TIM/DHAP and TIM in D_2O buffer, respectively. It is clear that without subtraction, the $\text{C}=\text{O}$ stretch band of the bound DHAP may not be identified. Figure 2c shows the difference spectrum between TIM/DHAP and TIM. The major $\text{C}=\text{O}$ band from bound DHAP is now apparent. However, a number of positive and negative bands with a higher IR intensity in the spectral region below 1700 cm^{-1} are also observed. They are due to frequency shifts of the protein bands upon substrate binding to the

enzyme, and it is a general feature in such difference vibrational spectroscopic studies (e.g., ref 18). As can be seen in Figure 2c, these bands are much stronger than the C=O bands of the bound DHAP. Thus, C=O/C=C stretch modes from other species in the reaction mixture, such as bound GAP or the enediol(ate) intermediate, may not be identified by this method if their frequencies are under the intense protein bands.

Isotope-Edited FTIR Difference Studies of the TIM/ Substrate Reaction Mixture at Equilibrium

To determine if the enediol(ate) intermediate or GAP exists in the TIM/ substrate reaction mixture, [2-¹³C]DHAP, [1,3-¹³C₂]DHAP, and [U-¹³C₃]DHAP were prepared and the IR difference spectra between TIM/DHAP and one of the TIM/¹³C-labeled DHAPs were obtained. When DHAP concentrations in these two samples were carefully matched, all protein bands were canceled out. Only those vibrational modes of the substrates that are affected by the ¹³C labeling will appear in such difference spectra. These bands may include C1=O (when [1,3-¹³C₂]DHAP was used) and C1=C2 stretch bands from GAP and enediol(ate), respectively, if they exist in significant amount. The exact match of the unlabeled and labeled DHAP concentrations is critical to prevent the much stronger protein IR bands (as shown in Figure 2c) from appearing in the difference spectrum. In our isotope-edited difference spectra, the vibrational modes from the unlabeled substrate appear as positive bands, whereas those from the ¹³C-labeled substrate appear as negative bands.

Figure 3a,b shows the difference spectrum between TIM/ DHAP (10 mN:10 mM) and TIM/[2-¹³C]DHAP (10 mN:10 mM) and between DHAP and [2-¹³C]DHAP in solution, respectively. In spectrum 3a, a small contribution (<10%) from free DHAP/[2-¹³C]DHAP was subtracted out. Because the C=O stretch bandwidth of the free DHAP is much broader than that of the bound DHAP (see Figure 3b), it is clear that the major positive band at 1714 cm⁻¹ and the minor band at 1732 cm⁻¹ in Figure 3a cannot be subtracted out, thus must be due to the bound DHAP. These two bands shift down by 38 and 33 cm⁻¹ upon ¹³C₂ labeling to 1676 and 1699 cm⁻¹, respectively, supporting the assignments of these two bands to the C2=O stretch mode of the TIM-bound DHAP.

Figure 3c,d shows the difference spectrum between TIM/ DHAP (10 mN:10 mM) and TIM/[1,3-¹³C]DHAP (10 mN:10 mM) and between DHAP and [1,3-¹³C]DHAP in solution, respectively. As expected, the isotope labeling of the C1, C3 carbons did not affect the DHAP C2=O stretch frequency; thus, the difference spectrum in solution was flat in this region (Figure 3d). Although the C1=O stretch band intensity is quite low in the GAP solution spectrum (see Figure 1a) due to low percentage of the GAP aldehyde form, the C1=O bond of GAP may be protected by the enzyme active site from hydration and thus observable in the IR spectrum of the TIM/ substrate reaction mixture, as shown in previous FTIR studies of several TIM mutants.^{16,17} 1,3-¹³C labeling of GAP should cause a 30–45 cm⁻¹ red shift of its C1=O stretch mode, resulting in a positive-negative pair in Figure 3c if GAP exists in significant amount in the reaction mixture. On the basis of the noise level in Figure 3c, we can estimate that the GAP population in the reaction mixture should be less than 5% of the DHAP population.

Because the C1=C2 bond in the expected enediol(ate) intermediate is not entirely symmetrical, its stretch mode should be IR-active and expected to show significant IR intensity in the 1640–1660 cm^{-1} region (e. g. Aldrich Library of FTIR Spectra) and should show a 30–40 cm^{-1} shift upon either 1- ^{13}C or 2- ^{13}C labeling. The absence of a positive-negative pair in this spectral region in both a and c of Figure 3 suggests that the population of the enediol(ate) intermediate in the reaction mixture is also very low.

Isotope-edited difference FTIR studies were also performed using [U- ^{13}C]DHAP. The results are virtually identical to those obtained using [2- ^{13}C]DHAP (Figure 3a), with no apparent negative bands due to the $^{13}\text{C}1=\text{O}$ and $^{13}\text{C}1=^{13}\text{C}2$ stretch modes from GAP and enediol(ate) intermediate (data not shown). Such results further support our conclusion that the GAP and enediol(ate) intermediate populations in the TIM/substrate reaction mixture are very low.

Substate Population Distribution within the DHAP Side of the Michaelis Complex in YeTIM and TcTIM

Our FTIR spectral pattern of YeTIM/DHAP is consistent with previously published FTIR results from different labs³⁸ and is virtually the same as the spectral pattern for the bound DHAP in chicken muscle.¹⁵ However, we noticed that although the substrate k_{cat} values for yeast and chicken muscle TIMs are very similar,^{33,38} they are about 3-fold different compared to the k_{cat} values in TcTIM.³⁹ One possible source for the difference in k_{cat} is that the on-enzyme substrate population distributions are differences in these TIMs from different organisms.

Figure 4a,b shows the difference FTIR spectra between TIM/DHAP and TIM/[2- ^{13}C]DHAP using YeTIM and TcTIM, respectively. For YeTIM, the IR intensity profile of the difference spectrum may be deconvoluted into a positive-negative pair of Gaussian-shaped bands using curve fitting, as shown in Figure 4a. For unlabeled DHAP, the intensity ratio of the 1714 cm^{-1} band and the 1731 cm^{-1} band is ~5.3:1. The bandwidths (full width at half-maximum) for the 1714 and 1732 cm^{-1} bands are 17 and 8 cm^{-1} , respectively. The narrower bandwidth of the 1732 cm^{-1} band suggests that the weakly hydrogen-bonded DHAP species is quite homogeneous, with very similar environments. The broader bandwidth of the 1714 cm^{-1} band suggests that the strong hydrogen-bonded DHAP species is somewhat heterogeneous because of different hydrogen bond strengths to the C2=O bond. 2- ^{13}C labeling of DHAP results in an isotope shift of 38 cm^{-1} of the 1714 cm^{-1} band to 1676 cm^{-1} . This shift is 5 cm^{-1} smaller than the isotope shifts of the solution DHAP C2=O stretch mode (Figure 1b,c), apparently due to increased delocalization of the DHAP C2=O stretch motion in the presence of multiple active site interactions. The minor 1732 cm^{-1} band shifts 33 cm^{-1} to 1699 cm^{-1} upon ^{13}C labeling. It is unclear what causes the smaller shift in this case, which is not observed in TcTIM (Figure 4b).

The 1714 cm^{-1} band in Figure 4a represents ~85% of YeTIM-bound DHAP species, which experiences strong hydrogen bonding to its C2=O bond, as demonstrated by its 14 cm^{-1} lower C2=O stretch frequency compared to that of the internally hydrogen-bonded DHAP species in solution (Figure 1b, 1728 cm^{-1} band). This frequency difference can be correlated to ~7 kcal/mol stronger hydrogen bonding to C2=O in the Michaelis complex to lengthen

this bond length by 0.007 Å.^{22–24} In the minor population of the bound DHAP (the 1732 cm^{−1} band), the hydrogen bond to its C2=O bond is weaker than that in the internally hydrogen-bonded DHAP in solution (Figure 1b, 1728 cm^{−1} band), by ~2 kcal/mol, as suggested by its 4 cm^{−1} higher C2=O stretch frequency. However, even for this bound DHAP form, the hydrogen bonding to its C2=O bond is still ~5 kcal/mol stronger than that in the DHAP in solution without the internal hydrogen bond (the 1742 cm^{−1} band in Figure 1b).

In clear contrast, the IR intensity profile of TcTIM-bound DHAP in the difference spectrum cannot be fitted to a two-peak curve because the major band around 1711 cm^{−1} is clearly not symmetrical (Figure 4b). One possible deconvolution of the IR intensity profile is shown in Figure 4b. The band at 1731 cm^{−1} in TcTIM is more isolated compared with the 1732 cm^{−1} band in YeTIM, and its bandwidth is 7 cm^{−1}, suggesting similar environments for the weakly hydrogen-bonded DHAP species in TcTIM compared to that in YeTIM. However, the ¹³C shift of this band is 43 cm^{−1}, the same as in solution. The major DHAP band may be curve-fitted by two bands, but one of them is extremely broad. Such results suggest that, unlike the case in YeTIM (and in chicken muscle TIM for that matter), the DHAP population distribution in TcTIM is not well defined. The hydrogen bonding strength to the DHAP C2=O bond varies wildly to yield a continuum C2 O stretch band in the FTIR spectrum. This continuum feature extends to a significantly lower frequency range to overlap with the 2-¹³C-labeled DHAP C2=O stretch mode region; thus, the band intensity and frequency are difficult to determine accurately. Nevertheless, on the basis of the results shown in Figure 4b, we can conclude that DHAP binds to TcTIM in at least two different forms: one is well-defined with weak hydrogen bonding to the C2=O bond and the other with strong hydrogen bond to the C2=O bond but with a wide range of bonding strength.

DISCUSSION AND CONCLUSIONS

Previous X-ray, NMR, and FTIR Studies on Substrate Population Distributions in the TIM Reaction Mixture Suggested a Single Linear Reaction Pathway

TIM has been the subject of various landmark spectroscopic studies elucidating the details of the mechanism in enzyme-catalyzed enolization chemistry. This enzyme provides a rare opportunity to studying the structure/dynamics of on-enzyme substrate structures in the enzyme/substrate Michaelis complex directly, unlike many other enzymes where similar studies can only be performed on an enzyme/inhibitor complex mimicking the Michaelis complex. For example, the structure of the DHAP-side TIM Michaelis complex at 1.2 Å resolution was determined by X-ray crystallography.¹³ The X-ray study revealed that the majority of the bound substrate is in the keto DHAP form, with the O1-C1-C2-O2 torsional angle in the range from 19° to 43°. The Nε of His95 forms a bifurcated hydrogen bond to O2 (2.6–2.7 Å) and O1 (3.0 Å) of the substrate, suggesting that the compact hydrogen bonding pattern at the active site should be relevant to promote the proton transfer along the reaction coordinate.¹³ The distance between Nε of His95 and O2 of DHAP is only 2.6–2.7 Å, indicating a strong hydrogen bond to DHAP C2=O to cause its polarization observed in FTIR studies. Apart from this strong hydrogen-bonded DHAP substate, there was no evidence for a weakly hydrogen-bonded DHAP substate structure based on the FTIR

studies. The study also suggested that, although not observed, the existence of a small amount of GAP or enediol(ate) reaction intermediate cannot be ruled out.¹³

The TIM/substrate reaction mixture at equilibrium with a uniformly ¹³C-labeled substrate was studied by solid-state and solution-state ¹³C NMR.¹² A single broad ¹³C resonance was found for the keto DHAP in the spectra under various conditions, indicating that all forms of keto DHAP, either free in solution or bound to TIM, are in fast exchange scheme in the NMR time scale. The ¹³C resonance from TIM-bound hydrated GAP was also identified and was estimated to be 5–10% of the ¹³C resonance from keto DHAP. The study also concluded that aldehyde GAP and enediol(ate) intermediate are not present on the enzyme in significant amount (<1%). These results provide a relatively complete picture of the substrate population distribution in the TIM reaction mixture at equilibrium. On the basis of the substrate population distribution, some important features related to the enzyme kinetics can also be reasonably speculated. For example, it was suggested that in the DHAP-to-GAP direction, all steps after the initial proton abstraction from DHAP are likely rapid, the reaction intermediate is likely short-lived, and the residence time of GAP on TIM is minimal.¹²

In the classical FTIR studies of the TIM/substrate reaction mixture at equilibrium, two C=O stretch bands were observed from TIM-bound DHAP, corresponding to two substates in the Michaelis complex.¹⁵ On the basis of such observation, it has been suggested that the reaction proceeds along a simple linear path, in which the keto DHAP first binds to TIM to form a weakly hydrogen-bonded substate (the inactive form) and then proceeds to another substate with a strong hydrogen bond to the C=O bond to become activated before proton abstraction from C1 of DHAP occurs.^{15,16,38}

NMR Evidence for Multiple Reaction Pathways

However, this simple linear reaction model was called into question by solution-state ¹H NMR studies on the product deuterium distributions for both GAP-to-DHAP and DHAP-to-GAP directions in D₂O.^{37,40} The results showed that the partitioning of the TIM/enediol(ate) intermediate between protonation at the carbonyl carbon and deuterium exchange with solvent was very different for the two starting substrates. Thus, the results provided the evidence that the reaction of these two substrates does not proceed through a single reaction intermediate but through distinct intermediates that differ in the bonding and arrangement of catalytic residues at the enediolate O1 and O2 oxyanions formed on deprotonation of GAP and DHAP, respectively.^{37,40} In other words, the TIM-catalyzed reaction does not follow a simple linear reaction pathway but rather two different reaction pathways depending on the direction of the reaction.

How Our Current Results Can Be Used To Determine if TIM-Catalyzed Reaction Follows Parallel Pathways

In our current study, simple enzymatic methods to form ¹³C-labeled DHAP at specific positions are reported. Isotope-edited FTIR difference measurements were conducted on the TIM/substrate reaction mixture at equilibrium to discover the C=O/C=C stretch band of the substrate/enediol(ate) intermediate masked by the intense protein amide band. The

results of our FTIR studies confirmed some of the main NMR findings on the substrate distribution in the Michaelis complex: the keto DHAP is the dominant form of the bound substrate; the populations of reaction intermediate enediol(ate) and aldehyde GAP are negligible. Although the hydrated DHAP and GAP are not detectable in our FTIR measurements due to low IR intensity of the C-OH vibrational modes, more details on the substrate population distribution of keto DHAP are determined. The weak C2=O band at a higher frequency near 1732 cm⁻¹ is quite sharp (bandwidth ~7–8 cm⁻¹) in both YeTIM and TcTIM. This band is due to a DHAP substate with weak hydrogen bonds to C2=O. The strong lower-frequency C2=O band near or below 1714 cm⁻¹ is either broad (bandwidth of 17 cm⁻¹) in YeTIM or a composite band, consisting of several bands, in TcTIM. This band is due to multiple DHAP substates with varying degrees of strong hydrogen bonding to C2=O.

In principle, the linear reaction scheme can still be consistent even with the more complex DHAP substate population distribution: keto DHAP binds to TIM with weak hydrogen bonding to C2=O to form the first substate along the reaction path; the hydrogen bonding to C2=O becomes progressively stronger to pass all observed strongly hydrogen-bonded DHAP substates in a linear fashion before proton abstraction from C1 of DHAP. However, there is another possibility that after reaching any substate of the strongly hydrogen-bonded DHAP each substate follows its own chemical pathway in a parallel fashion. This possibility was demonstrated by IR temperature-jump relaxation spectroscopic studies on a lactate dehydrogenase (LDH)/substrate reaction mixture at equilibrium.⁴¹ LDH catalyzes the interconversion of pyruvate and lactate, using NADH as a cofactor. It is possible to prepare the LDH/ substrate mixture such that half of the bound substrate is in the pyruvate side. The isotope-edited difference FTIR studies on this LDH reaction mixture showed that the C2=O intensity profile of the bound pyruvate can be fitted by four Gaussian curves, similar to that observed in the TIM reaction mixture shown in this study (Figure 4). One bound pyruvate C2=O band frequency is similar to that in solution, indicating a weak hydrogen bond, and the other three band frequencies are red-shifted by 24 to 36 cm⁻¹ relative to the solution value, suggesting three bound pyruvate substates with varying degrees of strong hydrogen bonding interactions to its C2=O bond by active site residues.⁴² A subsequent IR temperature-jump relaxation spectroscopy study on the LDH/pyruvate side of the Michaelis complex showed that the results can be best explained by the parallel reaction mechanism, in which each of the three strongly hydrogen-bonded pyruvate substates follows its own reaction path to the chemical step. Interestingly, the weakly hydrogen-bonded pyruvate substate was predicted not directly on the reaction path but rather in an end state of a branch from the main reaction path, possibly serving as a reservoir to regulate the substrate turnover rate.⁴³

Although TIM and LDH catalyze very different chemical reactions, they share a common mechanism to activate their respective substrate (DHAP and pyruvate) by initiating the polarization of the substrate C2=O bond with hydrogen bonding interactions from the active site His residue in the Michaelis complex. In the subsequent chemical step, proton abstraction from adjacent C1H₂OH of DHAP occurs in TIM but a hydride transfers from NADH to pyruvate C2 in LDH. The substate population distributions of the substrate in the Michaelis complexes are quite similar in these two enzymes, as shown by their C2=O IR

intensity profiles. However, one difference between LDH and TIM is that in LDH a correlation between the bond pyruvate C2=O frequency and k_{cat} of LDH exists in a series of LDH mutants,³¹ whereas no such correlation was observed in the FTIR studies of TIM mutants.^{16,17,33,34} This difference reflects the fact that in the chemical step, only C2 of pyruvate is involved in LDH, whereas both C1 and C2 of DHAP are involved in TIM. Nevertheless, our T-jump relaxation spectroscopic studies on the LDH reaction mixture at equilibrium have shown that the interconversion dynamics among the substates within the pyruvate side of the Michaelis complex can be determined. By characterizing DHAP substate interconversion dynamics in TIM, a future T-jump relaxation spectroscopic study will be able to determine if the TIM-catalyzed reaction follows a simple linear reaction pathway, as previously believed, or follows parallel reaction pathways, as suggested by the recent NMR study.

Acknowledgments

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ABBREVIATIONS

TIM	triosephosphate isomerase
DHAP	dihydroxyacetone phosphate
GAP	D-glyceraldehyde 3-phosphate
G3P	L-glycerol 3-phosphate
TEV	Tobacco Etch Virus Protease

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Figure 1.

(a) FTIR spectrum of DL-GAP (200 mM, pH 7 in D₂O). (b) FTIR spectrum of DHAP (100 mM, pH 7 in D₂O at room temperature). (c) FTIR spectrum of [2-¹³C]DHAP (100 mM, pH 7 in D₂O at room temperature). All samples were titrated to pH 7 without buffer, lyophilized, and then resuspended in D₂O. CaF₂ windows were used with a 15 μ m spacer. The solid lines are the experimentally determined spectra. The dashed lines are the results of curve fitting. The curve fittings were performed with program Igor with appropriate baseline correction when necessary. Both C1=O stretch bands from GAP and C2=O stretch bands from DHAP can be fitted with two peaks using Voigt line shape, indicating that more than

one conformations are present in the aldehyde form of GAP and the keto form of DHAP in solution.

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Figure 2.

a) FTIR spectrum of YeTIM/DHAP (10 mN:10 mM). (b) FTIR spectrum of YeTIM (10 mN). (c) a minus b and multiplied by 10. Enzyme samples are in 50 mM tris, 50 mM NaCl at pH 7.8 (meter reading) in D₂O at room temperature. CaF₂ windows were used with a 15 μ m spacer. YeTIMs exist as dimers in solution, and the concentration unit (mN) used here reflects the TIM active site (subunit) concentration. Enzyme samples were prepared as described in the Methods section.

Figure 3.

(a) Difference spectrum between YeTIM/DHAP (10 mN:10 mM) and YeTIM/[2- ^{13}C]DHAP (10 mN:10 mM). (b) Difference spectrum of DHAP (100 mM) and [2- ^{13}C]DHAP (100 mM). The intensity was scaled to match the spectrum in (a). (c) Difference spectrum between YeTIM/DHAP (10 mN:10 mM) and YeTIM/[1,3- $^{13}\text{C}_2$]DHAP (10 mN:10 mM). (d) Difference spectrum of DHAP (100 mM) and [1,3- $^{13}\text{C}_2$]DHAP (100 mM) with adjusted intensity as in (b). All enzyme samples were in 50 mM tris, 50 mM NaCl at pH 7.8 (meter reading) in D_2O at room temperature. DHAP samples were adjusted to pH 7 without buffer in D_2O . CaF_2 windows were used with a 15 μm spacer.

Figure 4.

(a) Difference spectrum between YeTIM/DHAP (10 mN:10 mM) and YeTIM/[2-¹³C]DHAP (10 mN:10 mM) at room temperature. (b) Difference spectrum of TcTIM/DHAP (10 mN:10 mM) and TcTIM/[2-¹³C]DHAP (10 mN:10 mM) at room temperature. The sample conditions were the same as described in Figure 3. The solid lines are the experimentally determined spectra. The dashed lines are the results of curve fitting. The curve fittings were performed with program Igor. Gaussian line shapes were used in the curve fitting.

Scheme 1.