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Active-Site Glu165 Activation in Triosephosphate Isomerase and Its Deprotonation Kinetics

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Abstract

Triosephosphate isomerase (TIM) catalyzes the interconversion between dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP) via an enediol(ate) intermediate. The active-site residue Glu165 serves as the catalytic base during catalysis. It abstracts a proton from C1 carbon of DHAP to form the reaction intermediate and donates a proton to C2 carbon of the intermediate to form product GAP. Our difference Fourier transform infrared spectroscopy studies on the yeast TIM (YeTIM)/phosphate complex revealed a C=O stretch band at 1706 cm⁻¹ from the protonated Glu165 carboxyl group at pH 7.5, indicating that the p*K*_a of the catalytic base is increased by >3.0 pH units upon phosphate binding, and that the Glu165 carboxyl environment in the complex is still hydrophilic in spite of the increased p*K*_a. Hence, the results show that the binding of the phosphodianion group is part of the activation mechanism which involves the p*K*_a elevation of the catalytic base Glu165. The deprotonation kinetics of Glu165 in the μs to ms time range were determined via infrared (IR) T-jump studies on the YeTIM/phosphate and (“heavy enzyme”) [U-¹³C, -¹⁵N]YeTIM/phosphate complexes. The slower deprotonation kinetics in the ms time scale is due to phosphate dissociation modulated by the loop motion, which slows down by enzyme mass increase to show a normal heavy enzyme kinetic isotope effect (KIE) ~1.2 (i.e., slower rate in the heavy enzyme). The faster deprotonation kinetics in the tens of μs time scale is assigned to temperature-induced p*K*_a decrease, while phosphate is still bound, and it shows an inverse heavy enzyme KIE ~0.89 (faster rate in the heavy enzyme). The IR static and T-jump spectroscopy provides atomic-level resolution of the catalytic mechanism because of its ability to directly observe the bond breaking/forming process.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.9b02981.

Active site cavities in TIM/DHAP and TIM/phosphate complexes and phosphate concentration-dependent TIM Trp emissions with Hill equation analysis (PDF)

The authors declare no competing financial interest.

INTRODUCTION

Triosephosphate isomerase (TIM) catalyzes the interconversion of dihydroxy acetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP) by an enolization mechanism involving a cis-enediol intermediate. The simplest reaction mechanism depicted in Scheme 1 was proposed by Alberty and Knowles.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 DHAP C1 carbon by the active site Glu165 carboxylate to form the enediol(ate) reaction intermediate, with possible concomitant proton transfers from His95 to C2 O oxygen and from C1—OH to His95. In the next step, a proton transfers from Glu165 carboxyl to C2 carbon to form the product GAP to complete the reaction.

The TIM/substrate reaction mixture has been studied extensively by X-ray crystallography,² NMR,^{3,4} and Fourier transform infrared spectroscopy (FTIR).^{5,6} All these studies show that, in the wild-type enzyme near neutral pH, TIM/ DHAP is the predominate species. No detectable GAP or reaction intermediate (with a protonated Glu165) can be observed in the TIM/substrate complex except in certain mutant TIMs where GAP or protonated carboxyl group from Glu165 may be observed by FTIR.^{7–9}

The reaction mechanism described in Scheme 1 indicates that the pK_a of the Glu165 carboxyl and the pK_a of the DHAP C1 deprotonation should become comparable at the time of the proton transfer as the reaction progresses along the reaction coordinate toward the transition state. The pK_a of a Glu carboxyl group in solution is 3.9 and it remains the same for Glu165 in unligated yeast TIM (YeTIM).¹⁰ On the other hand, the pK_a for deprotonation of DHAP at C1 in solution was reported to be 18, ~14 pH units higher than Glu carboxyl.¹¹ Therefore, progressive increase of Glu165 pK_a along the reaction coordinate toward the transition state is expected to be an important component for enzyme activation. It has been suggested that the substrate binding anchored by its phosphodianion moiety creates an active site pocket with restricted access of water to Glu165, and this reduction in solvation accounts for the free energy necessary to raise the pK_a of Glu165 in the Michaelis complex by two pH units.¹² In the transition-state analogues formed between phosphoglycolate

trianion and TIMs from several different sources, the pK_a of the catalytic base (Glu165 or its equivalent) is raised by more than 6 pH units to 10.4–10.9.^{13,14}

More recent studies demonstrated that the phosphodianion moiety of the substrate also provides differential energies in ground and transition states to reduce the reaction barrier. This was elegantly shown in the TIM-catalyzed hydrogen exchange reactions on the truncated substrate glycolaldehyde, using phosphite as an activator.¹⁵ These studies determined that the binding of phosphite reduced the ground-state energy by 1.9 kcal/mol but by 5.8 kcal/mol in the transition state, thus reduced the transition barrier for the C—H proton exchange reaction on the glycolaldehyde C2 carbon by 3.9 kcal/mol. The study attributed the phosphite activation of the enzyme to the population increase of the loop-closed conformation. In a recent study using the transition-state analogue complexes formed between phosphoglycolate and a number of TIM mutants, it was found that there is a strong correlation between the pK_a of the active site Glu and the k_{cat}/K_M of the TIM mutants.¹⁴

Our current difference FTIR studies show that the binding of dianionic phosphate to the YeTIM active site increases the pK_a of the active site Glu165 residue by >3.0 pH units to >7.0, higher than Glu165 pK_a in the Michaelis complex. Furthermore, the pK_a increase is not associated with the hydrophobic environment near the Glu165 carboxyl group. In fact, the hydrogen bond to the Glu165 carboxyl C=O bond in the TIM/phosphate complex is stronger than that of acetic acid in aqueous solution. Our infrared (IR) T -jump studies on the TIM/phosphate complex discovered at least two distinct kinetic processes for the deprotonation of the Glu165 carboxyl group. The sub-millisecond to millisecond kinetics are due to phosphate dissociation, consistent with the similar loop open—close kinetics in TIM or TIM/inhibitor complexes determined by previous NMR and fluorescence T -jump studies.^{16–19} The tens of μ s Glu165 deprotonation kinetics, not detected in previous fluorescence T -jump studies, are believed to be associated to the temperature-induced Glu165 pK_a change, which presumably is related to certain protein conformational changes in the TIM/phosphate complex.

Finally, the effects of protein mass modulation on the thermodynamics and dynamics of phosphate binding and Glu165 deprotonation were investigated by using [U-¹³C,-¹⁵N]-labeled TIM. To investigate possible coupling of protein motions to the barrier crossing in the chemical bond breaking/formation step of the enzyme-catalyzed reaction, a research technique based on the “heavy enzymes” kinetic isotope effect (KIE) has been developed in Schramm’s lab²⁰ and later applied to a number of enzyme systems by other researchers.^{21–26} One hypothesis assumed in such studies is that isotopic substitution of nonexchangeable atoms by their heavy isotope counterparts would slow down protein pico-second to femtosecond dynamics without any effect on system’s electrostatics following the Born—Oppenheimer approximation (for a recent review, see ref 27). While the protein’s fast dynamics may indeed be reduced in “heavy” enzymes in a way that reduces the probability of barrier crossing of its “chemical step”, it may not be the general rule as pointed out in a recent review.²⁸

One important consideration in these heavy enzyme KIE studies is to find an approach to determine the kinetics for the true (or intrinsic) “chemical step”, which may typically be

described by a single rate constant obtained from presteadystate single turnover measurements or by steady-state kinetic studies coupled with forward commitment factor measurements (e.g., see recent reviews^{27,29}). Our IR *T*-jump relaxation studies on the Glu165 deprotonation process in the TIM/ phosphate complex revealed that it cannot be described by a single kinetic rate. The implications of our observations on the enzyme dynamics and function relationships will be discussed.

MATERIALS AND METHODS

DHAP was purchased from Sigma. 2-¹³C labeled DHAP was synthesized by enzymatic methods described previously.⁶ The YeTIM gene was synthesized and subcloned to the pET23a+ plasmid by General Biosystems. 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 amino acid sequence ENLYFQS to create the cleavage point for tobacco etch virus protease (TEV). The entire DNA sequence added to the 5' end of the YeTIM gene was ATGGGTAGCTCTCATCATCACCAT-CATCACAGCAGCGGCGAGAACCTGTATTTTCA-GAGCGCCGCG (corresponding to amino acid sequence MGSSHHHHHSSGENLYFQSAA). The pET23a+ plasmid with the YeTIM gene was transformed into the *Escherichia coli* BL21(DE3) cell line. Unlabeled or uniformly [U-¹³C, -¹⁵N] labeled YeTIM were prepared from the *E. coli* cells cultured in 2*YT or minimal medium supplemented with 2 g of U¹³C glucose and 0.5 g ¹⁵N NH₄Cl per L medium, following the published procedures.^{6,30} 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 on to 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 M NaCl/20 mM imidazole, the YeTIM with the His tag was eluted by 0.5 M NaCl/200 mM imidazole from the Ni⁺ column. The His tag of the YeTIM was removed by mixing with TEV (also with His tag) at a 50:1 ratio in a dialysis bag and dialyzed for 2 h at room temperature with three changes of the 50 mM Tris buffer at pH 7.5 and then overnight at 4 °C. The TEV/YeTIM reaction mixture was then passed through the Ni⁺ column to collect the YeTIM without the His tag. The purity of thus purified YeTIM was checked with sodium dodecyl sulfate gel and was typically >90% pure. The typical yield of the [U-¹³C, -¹⁵N] YeTIM protein was >15 mg/L by this procedure. The concentrations of the YeTIM sample were determined by UV—vis using molar extinction coefficient $\epsilon_{280} = 25.4 \text{ cm}^{-1} \text{ mM}^{-1}$.

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/phosphate and TIM sample solutions were simultaneously loaded into a dual-cell shuttle accessory. Two CaF₂ windows with a 25 μm Teflon spacer sandwiched in between were used to hold the sample. Typical sample volume was 5 μL . Spectra were collected in the range of 1100–4000 cm^{-1} with 2 cm^{-1} resolution. Blackman—Harris three-term apodization and Happ-Genzel apodization were applied, respectively. OmMic 7.1 (Nicolet Instruments, Corp.) software was used for data collection and analysis.

IR temperature-jump (T -jump) relaxation spectroscopy measurements were performed with a setup similar to that described previously.^{31–33} Briefly, the fundamental 1064 nm line from a Nd:YAG laser (250 mJ pulse, 5 Hz repetition rate, 7 ns pulse width, Quantel 980 Quantel, France) was focused into a homemade Raman shifter cell filled with H₂ gas at 700 psi. The output beam from the Raman shifter was then dispersed by a Pellin—Broca prism, and the 1.91 μ m line was picked by a pin hole and then split by a 50–50 beam splitter into two counter-propagating pump beams. These two pump beams were introduced through the same volume of the sample from the opposite sides of the IR cell, allowing for uniform heating of the sample solution prepared in D₂O. The heated region of the sample was probed by a tunable quantum cascade laser (Daylight Solutions Inc., Poway, CA) with ~ 5 cm⁻¹ frequency increment in the 1700–1765 cm⁻¹ range for unlabeled YeTIM (model TLS-41058) and in the 1660–1700 cm⁻¹ range for [U-¹³C, -¹⁵N] YeTIM (model TLS-41060). The exact probe frequencies were adjusted to avoid water vapor lines from the atmosphere. Relaxation changes after the T -jump pulse in probe beam transmission were detected by a MCT detector with integrated preamplifier (Kolmar Technologies, Newburyport, MA). The signal from the MCT detector was digitized by a Tektronix DPO3052 oscilloscope 8-bit digitizer with 10 ns sampling intervals.

The IR T -jump cell consists of two stacked CaF₂ windows that are separated by a 260 μ m Teflon spacer split into two compartments, one for the sample and the other for the reference. Using this design, reference data can be collected under nearly identical conditions as the sample data. The cell was mounted on a cell holder with its temperature controlled by a water bath. The positions of the cell holder were controlled by a two-dimensional translational stage interfaced to a computer.

A home-written Labview program was used to control the IR laser frequency/power level, sample temperature, sample cell position, and data acquisition from the oscilloscope. Typical temperature jumps in the range of 5–18 °C may be achieved. The useful time range of the instrument in this study is from μ s to 2.3 ms. The fast time limit is set by the bandwidth of the preamplifier of the detector. The slow time limit is set by the data transfer time of ~ 0.16 s (for data recorded within 2.3 ms) from the oscilloscope to PC to allow the 5 Hz repetition rate. The geometry of our sample cell arrangement is such that heat diffuses out of the irradiated volume with a lifetime of a few tens of ms to allow nearly full recovery of the sample temperature after each T -jump pulse.

All enzyme samples in this study were prepared in 0.1 M Tris, 0.05 M NaCl buffer in D₂O at pH 7.5 (pH meter reading). Before experiments, the sample was washed in a Centricon concentrator with the buffer four times at 10 $\times$ dilution and stored at 4 °C=Overnight. The typical concentration of the stock TIM solution was 10 mM. 1.0 M phosphate stock solution in D₂O at pH 7.5 was prepared for fluorescence phosphate titration studies.

RESULTS AND DISCUSSION

Phosphate Binding-Induced TIM Structural Changes Detected by FTIR.

Figure 1a shows the FTIR spectra of the YeTIM and YeTIM/phosphate complex, respectively, in the C=O stretch frequency region. The main band in this region is due to the

amide I band, which is sensitive to the local protein secondary structures and hydrogen bonding strength on the amide C=O bond (e.g., see ref 34). The perturbation on the TIM structure upon phosphate binding is readily revealed by their difference spectrum as shown Figure 1b. A subtraction factor of 0.96 was applied to compensate for the slight intensity mismatch of the protein band intensities, so that the baseline of the difference spectrum is flat. The negative band near 1677 cm^{-1} in Figure 1b may be assigned to amide I from the random coil structure in the apo YeTIM.³⁴ Upon binding of phosphate, this amide band shifts to either higher frequency near 1693 cm^{-1} (with larger distortion of the amide HN—C(=O) bond often found in the β -turn structure) or lower frequency near 1640 cm^{-1} (with stronger hydrogen bonding to amide C=O. e.g., see ref 35). Thus, the enzyme spectral change induced by phosphate binding is consistent with an enzyme structural change involving a random coil (the catalytic loop) becoming more ordered upon substrate binding. In the difference FTIR spectrum between YeTIM/ DHAP and YeTIM shown in Figure 1c, the spectral change for DHAP binding is more extensive but contains the same feature as in Figure 1b: a positive band near 1695 cm^{-1} and a negative band near 1677 cm^{-1} . This suggests that DHAP interacts with TIM to cause more perturbations on the protein structure in addition to the random coil change. This interpretation of the FTIR results is consistent with X-ray crystallographic studies. In both X-ray structures of TIM/phosphate and TIM/ DHAP complexes, the phosphate group binds to the same enzyme residues to bring the catalytic loop to a closed conformation to form an active site cavity. The catalytic base Glu165 is located at the bottom of the cavity (see Figure S1).^{2,36,37}

Detection of the Carboxyl C=O Stretch Band in the YeTIM/Phosphate Complex.

Typically, the C=O stretch modes in carboxyl or ketone groups have higher frequencies than that in amide. Thus, the difference spectrum between the TIM/substrate complex and TIM may reveal the C=O stretch modes from the substrate, as demonstrated in the original TIM/ DHAP and TIM difference FTIR studies,⁵ or from the protonated carboxyl group in TIM, as demonstrated in FTIR studies of several TIM mutant/substrate complexes.^{7–9} Figure 2a shows the C=O stretch frequency region of the difference FTIR spectrum between the TIM/ substrate reaction mixture (which can be formed by adding stoichiometric amount of DHAP to concentrated TIM solution) and TIM in 0.1 M Tris buffer with 0.05 M NaCl at pH 7.5 (uncorrected pH meter reading) in D_2O . The band labeled with ** is due to protein amide C=O stretches that shifted from lower frequencies upon DHAP binding as discussed above. The two bands at 1713 and 1732 cm^{-1} vanished upon ^{13}C labeling of the substrate as shown in Figure 2b, and thus can be assigned to the bound DHAP. The 1713 cm^{-1} band is red-shifted by about 20 cm^{-1} relative to the average solution DHAP C2=O stretch band and was suggested to be the active form of the bound substrate due to its more polarized C2=O bond, and presumably part of the DHAP C1–H bond activation mechanism.⁵ Previous NMR and difference FTIR studies also ruled out significant population of the enediol intermediate in the wild-type TIM reaction mixture.^{4,6}

The C=O stretch mode from a protonated carboxyl group in a protein is typically in the spectral region $>1700\text{ cm}^{-1}$, which is absent in the YeTIM/ ^{13}C -labeled substrate complex (Figure 2b). Thus, there is no evidence for a protonated carboxyl group in the wild-type TIM/DHAP Michaelis complex near neutral pH. This is consistent with a $\text{p}K_{\text{a}}$ of ~ 6 of the

active site Glu165 as determined from pH-dependent enzyme kinetic studies.¹² The C=O bands from the protonated enzyme carboxyl group were observed in a number of FTIR studies on several TIM mutants,^{7–9} suggesting the existence of the reaction intermediates in those enzyme/substrate reaction mixtures. Interestingly, in the difference spectrum between TIM/phosphate and TIM, there is a new band at 1706 cm⁻¹ besides the protein amide I band labeled with ** (Figure 2c). We can assign this C=O band to a protein residue with a protonated carboxyl group because the phosphate vibrational modes in any ionic states are <1300 cm⁻¹.³⁸ The phosphate binding to TIM apparently increased the p*K*_a of this carboxyl by >3.0 pH units to >7.0.

Environment of the 1706 cm⁻¹ Carboxyl C=O Stretch Band in the YeTIM/Phosphate Complex.

It is generally assumed that the protein carboxyl p*K*_a increase may be attributed to the desolvation near the carboxyl group (e.g., see ref 39). The carboxyl C=O stretch frequency is typically >1730 cm⁻¹ in a number of TIM mutants complexed with the substrate as determined by FTIR,^{7,8,40} or in other enzymes.⁴¹ These frequencies are much higher than the C=O stretch mode of the typical carboxyl C=O band in aqueous solution. For example, there are two C=O bands from acetic acid in D₂O. The major band is at 1712 cm⁻¹ and a shoulder band is at 1685 cm⁻¹. The 1685 cm⁻¹ band intensity decreases with temperature increase and virtually disappears at >90 °C, thus it can be assigned to the C=O stretch from the dimeric complex. The 1712 cm⁻¹ band can be assigned to the monomeric acetic acid C=O stretch (data not shown). Therefore, the carboxyl groups with the C=O stretch band >1730 cm⁻¹ in those TIM mutants/substrate complexes are mostly desolvated and in hydrophobic environments.

In general, C=O stretch frequency is sensitive to electrostatic interactions, including ionic interaction with external ions, hydrogen bonding interaction, and polar dipole—dipole interaction, with the C=O bond. There have been two general approaches to describe the electrostatic interactions on the C=O bond quantitatively in terms of C=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.⁴² The relationship between C=O stretch frequency and interaction energy of a simple C=O containing molecule interacting with various cations and water has been investigated by ab initio calculations, and it was found that the shift in 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.⁴³ These computational results agree remarkably well with the experimentally determined correlation between C=O stretch and enthalpy of hydrogen bond formation on similar simple ketones observed in a smaller energy range (<10 kcal/mol).⁴⁴ For more complex cases, such as the C=O stretch frequency in an amide, the interaction energy may still be determined with additional computational analysis.³⁵

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.⁴⁵

Because the protein carboxyl C=O frequencies in TIM mutants/substrate complexes are more than 20 cm⁻¹ higher than that of the C=O stretch of acetic acid in aqueous solution, the interactions of the carboxyl with its environment is >10 kcal/mol weaker, indicating a mostly desolvated or hydrophobic environment.

On the other hand, the C=O stretch band in the TIM/ phosphate complex at 1706 cm⁻¹ is 6 cm⁻¹ lower than that of the acetic acid monomer in aqueous solution (data not shown), suggesting hydrogen bonding to carboxyl C=O is ~3 kcal/mol stronger than that of the acetic acid monomer in aqueous solution. Figure 3 shows the active site water molecules/ions around Glu165 (or its equivalent) in the TIM/DHAP complex (a. pdb1NEY²) and in TIM/phosphate complexes (b. pdb4POC³⁶ and c. pdb4Y96³⁷). Based on these X-ray structures, four structural water molecules form a hydrogen bonding network with one of the Glu165 carboxyl oxygens in a zig-zag pattern (Figure 3). The O—O distances in this network are between 2.55 and 2.80 Å, well positioned for proton transfers to and from the Glu165 carboxyl oxygen as summarized in a recent review on the proton transfer processes among water molecules in aqueous solution.⁴⁶ In addition, an ion is trapped between phosphate and Glu165 (or its equivalent) in the TIM/phosphate complex and interacts strongly with the other oxygen of the carboxyl (distances 2.94–3.14 Å, Figure 3b,c). In the human TIM/phosphate complex (4POC), the ion was assigned to Br⁻³⁶ but in the *Gemmata obscuriglobus* TIM/phosphate complex (4Y96), the ion was assigned to Na⁺.³⁷ Because of the dianion nature of the bound phosphate,^{47,48} we believe the trapped ion is most likely a cation so that the repulsive interaction with the bound phosphate can be avoided. Our observation of a C=O band at 1706 cm⁻¹ in Figure 2c is consistent with a trapped cation in the TIM/phosphate complex near Glu165 carboxyl C=O bond: the water hydrogen bonding network provides the proton to one Glu165 oxygen to form a C—OH group, and the cation interacts with the C=O oxygen to reduce its stretch frequency below that in aqueous solution. Thus, our difference FTIR results show that the carboxyl group pK_a increase in protein is not necessarily associated to the hydrophobic environment, as in a number of other studies.^{7,8,40,41} While it is surprising that Glu165 pK_a increase is larger in the TIM/phosphate complex than in the TIM/DHAP complex, it is consistent with the suggestion by Richards group that the phosphodianion moiety of the substrate is the driving force in activating the enzyme for catalysis, not only in TIM but also in other enzymes.⁴⁷

Binding Affinity of Phosphate to YeTIM by Trp Fluorescence.

Phosphate is a known inhibitor to TIM-catalyzed reactions, with K_i of ~20 mM.¹⁵ Upon binding of phosphate, the catalytic loop closes to change the environment of Trp168 located at the hinge of the loop.^{49,50} Therefore, the emission of Trp168 is sensitive to phosphate binding and has been used to study the loop dynamics in ligand binding.¹⁷ The phosphate concentration-dependent Trp emission at pH 7.5 in D₂O was determined. The emission profile starts with an increasing intensity phase, followed by a decreasing intensity phase and then becomes concentration-independent at high phosphate concentrations (see Figure

S2). The increase of the YeTim fluorescence intensity is half way toward its maximum when phosphate concentration reaches ~ 75 mM. In our subsequent studies, we use phosphate concentrations near this value to ensure that the Glu165 carboxyl C=O band intensities in the YeTIM/phosphate complexes are sufficient for FTIR studies and for Glu165 deprotonation kinetic studies by IR T -jump measurements. Detailed analysis on the phosphate binding data, as well as alternative interpretations on the phosphate binding mechanism other than ionic strength-dependent K_d values suggested in a previous study¹⁰ may be found in the Supporting Information.

Heavy Protein Effects on the Phosphate Binding.

The phosphate concentration-dependent Trp emission in $[U-^{13}C, -^{15}N]$ -labeled YeTIM was determined, and the results were analyzed using the Hill equation. There were no detectable difference in the active-site binding phase in $[U-^{13}C, -^{15}N]$ -labeled and unlabeled YeTIM (see Figure S2 in the Supporting Information). However, there are subtle differences in the nonspecific binding phase of the phosphate titration curves, suggesting that the enzyme mass change may have caused certain changes in the enzyme structure or dynamics that may be detected by other spectroscopy methods.

Figure 4 shows the difference spectrum between YeTIM/ phosphate and YeTIM, along with the curve fitting results in the C=O stretch region. The C=O stretch of the Glu165 carboxyl group is assigned to the band at 1706 cm^{-1} , which is 23 cm^{-1} higher than the enzyme amide I band at 1693 cm^{-1} . The Glu165 carboxyl C=O stretch bandwidth determined from curve fitting using a Voigt profile is 6.2 cm^{-1} . Figure 5 shows the difference spectrum between $[U-^{13}C, -^{15}N]$ YeTIM/ phosphate and $[U-^{13}C, -^{15}N]$ YeTIM, along with the curve fitting results in the C=O stretch region. The $^{13}C=O$ stretch of the Glu165 carboxyl group is assigned to the band at 1663 cm^{-1} , which is also 23 cm^{-1} higher than the enzyme amide I band at 1650 cm^{-1} . The Glu165 carboxyl $^{13}C=O$ stretch bandwidth determined from the curve fitting using a Voigt line shape is 8.0 cm^{-1} . The wider $^{13}C=O$ bandwidth suggests a wider interaction energy distribution between the Glu165 $^{13}C=O$ bond and its surroundings in the $[U-^{13}C, -^{15}N]$ YeTIM/phosphate complex compared to the unlabeled YeTIM complex.

Temperature Dependence of the Glu165 C=O Stretch Band in the YeTIM/Phosphate Complex.

Figure 6 shows the difference spectra of YeTIM/phosphate and YeTIM at seven different temperatures from 5 to 35 °C in the spectral region $>1690\text{ cm}^{-1}$. The intensity decrease of the 1706 cm^{-1} band upon temperature increase is due to the deprotonation of the active site carboxyl group, which may be caused by temperature-induced phosphate dissociation from the binary complex and/or the Glu165 pK_a change. The intensity of the protein amide band at 1693 cm^{-1} also decreases in proportion with the 1706 cm^{-1} band upon temperature increase, reflecting the dissociation of the phosphate at higher temperatures. Figure 7 shows the difference spectra of $[U-^{13}C, -^{15}N]$ YeTIM/phosphate and $[U-^{13}C, -^{15}N]$ YeTIM at seven different temperatures from 5 to 35 °C in the spectral region $>1645\text{ cm}^{-1}$. The intensities of the 1663 cm^{-1} band at different temperatures show similar changes compared to those of the unlabeled enzyme and the widths of the bands are consistently broader than the unlabeled Glu165 1706 cm^{-1} bands. The arrows in spectrum b in Figures 5 and 6 are the IR probe

frequencies used in the IR T -jump relaxation spectroscopy studies described below. These frequencies are not evenly spaced to avoid the water vapor lines in the atmosphere.

Deprotonation Kinetics of Glu165 Carboxyl by IR T -Jump Relaxation Measurements.

The inset in Figure 8 shows the IR T -jump relaxation transients probed at 1710 cm^{-1} on TIM/phosphate and TIM, respectively, and their difference. A 7 ns laser pulse jumps the solvent temperature, and the absorbance of the enzyme sample at 1710 cm^{-1} was measured by a continuous IR beam to monitor the relaxation of the sample which re-equilibrates toward the new elevated temperature. The main signal intensity drop after the T -jump pulse is due to the solvent D_2O temperature jump, and IR detector's response time ($\sim 0.5\text{ }\mu\text{s}$) determines the fast time measurements limit of our relaxation transient. Our observations on the relaxation process terminated at 2.3 ms because of combined constraints from the data transfer time and sample cooling (time constant $\approx 20\text{ ms}$ under our experimental conditions). To determine Glu165 deprotonation kinetics, we used difference transients between YeTIM/phosphate and YeTIM, which eliminates the relaxation signals from solvent and from most of other enzyme amide C=O bands in the spectral region $>1700\text{ cm}^{-1}$. The initial drop of the relaxation signal is about 120 mOD, corresponding to $\sim 15\text{ }^\circ\text{C}$ temperature increase. The final temperature after the T -jump was $\sim 25\text{ }^\circ\text{C}$.

The Glu165 deprotonation kinetics observed in our T -jump measurements on the TIM/phosphate complex is due to proton transfer from Glu165 to active-site structural water molecules shown in Figure 3. We believe this proton transfer is a part of the proton relay network in the TIM-catalyzed isomerization, and it can also explain the fact that the protonated Glu165 is only partially equilibrated with solvent in the TIM/substrate complex.
51,52

The main transients in Figure 8 show the difference IR T -jump relaxation kinetics between TIM/phosphate and TIM at 10 probe frequencies (dashed lines) as shown in Figure 6b. The relaxation transient observed for the 1698 cm^{-1} IR probe is nearly a straight line across the entire time range and cannot be curve-fitted to 3 or even 4 exponentials; apparently, it contains kinetics from protein bands due to altered frequencies by phosphate binding. Thus, this transient is omitted in Figure 8. The relaxation curves with probe frequencies at 1725 cm^{-1} or higher are basically flat as expected because protein absorbance in this frequency region is quite low and apparently does not change much with phosphate binding. The relaxation transients probed at 1704 and 1710 cm^{-1} , which are -2 and $+4\text{ cm}^{-1}$ from the center of the 1706 cm^{-1} Glu165 carboxyl C=O band, respectively, may be reasonably fitted by three exponentials as shown in the figure (solid lines). The slowest rates in these transients are in the $1300\text{--}1600\text{ s}^{-1}$ range, associated with the largest amplitudes. However, it is apparent that the relaxation process clearly continues beyond the 2.3 ms time limit of our spectra. In fact, the observed amplitude changes within 2.3 ms are only $\sim 75\%$ of the absorbance change estimated from the static FTIR measurements with comparable temperature change (see Figure 6). The faster rates in the relaxation transients are in the $8000\text{--}9000\text{ s}^{-1}$ range, but with lowest amplitudes. The fastest rates in these two relaxation transients are in the $300\text{--}360\text{ }000\text{ s}^{-1}$ range, with amplitudes of about 1/5 of the slowest phase. The relaxation transients probed at 1714 and 1721 cm^{-1} , which are $+8$ and $+15\text{ cm}^{-1}$

from the center of the 1706 cm^{-1} Glu165 carboxyl C=O band, respectively, may be fitted by two exponentials (solid lines). The relaxation rates are in the $280\,000\text{--}300\,000\text{ s}^{-1}$ range for the fast phase and in the $2400\text{--}2900\text{ s}^{-1}$ range for the slow phase. Table 1 shows the relaxation rates based on curve fitting of the relaxation transients probed by 1704 , 1710 , and 1714 cm^{-1} , where the amplitudes are relatively large. Although the observed kinetics in our relaxation transients is due to deprotonation of Glu165 carboxyl, we may relate these three kinetic rates to previously identified physical kinetic processes in this enzyme.

Glu165 Carboxyl Deprotonation Kinetics due to Phosphate Dissociation.

In previous fluorescence T -jump relaxation measurements on the YeTIM/glycerol-3-phosphate (G3P) complex, it has been shown that the loop open and close rates associated with G3P binding are ~ 2500 and $47\,000\text{ s}^{-1}$, respectively, at $25\text{ }^{\circ}\text{C}$.¹⁷ The fluorescence T -jump relaxation measurements on the YeTIM/phosphate complex showed a more complex relaxation pattern, including similar relaxations in the $50\,000\text{ s}^{-1}$ range and in 1000 s^{-1} . However, no relaxation kinetics faster than $100\,000\text{ s}^{-1}$ was observed in any of the TIM/ligand complexes.¹⁷ NMR studies on various TIM/ligand complexes showed the exchange rates between loop-open and loop-closed conformations are from 3000 to $12\,000\text{ s}^{-1}$.^{16,18,19,53} Therefore, it is reasonable to assign the two slower kinetic processes with $8000\text{--}9000\text{ s}^{-1}$ and $1300\text{--}1600\text{ s}^{-1}$ rates in our IR T -jump relaxation transients probed at 1704 H and 1710 cm^{-1} , and the $2400\text{--}2900\text{ s}^{-1}$ rates in relaxation transients with 1714 and 1721 cm^{-1} probes to the deprotonation of Glu165 caused by the loop motion- modulated phosphate dissociation.

Glu165 Deprotonation due to Temperature-Induced pK_a Change.

Because the faster kinetics ($>300\,000\text{ s}^{-1}$) observed in our IR T -jump relaxation transients cannot be explained by phosphate dissociation, it must be associated with the temperature-induced protein structural variations that caused the deprotonation of Glu165 carboxyl or the temperature-induced pK_a decrease when phosphate is still bound. This suggestion is consistent with recent NMR relaxation studies on protonation/deprotonation kinetics of 11 carboxyl groups from Asp/Glu residues in a small protein. The NMR results showed that the proton off rates of these carboxyl groups are in the range of $100\,000\text{--}3\,000\,000\text{ s}^{-1}$, and are weakly correlated to the pK_a of the carboxyl groups. In particular, one Asp carboxyl with elevated pK_a near 6.4 showed a k_{off} rate of $\sim 100\,000\text{ s}^{-1}$, and its pK_a decreases by $\sim 0.1\text{ pH}$ unit with $10\text{ }^{\circ}\text{C}$ temperature increase from 15 to $25\text{ }^{\circ}\text{C}$.⁵⁴ Thus, we may relate the fastest rates observed in our IR T -jump relaxation transients, which fall into the NMR- determined proton off-rate range of carboxyl groups in protein, to a temperature-induced Glu165 pK_a change. The average rate for the deprotonation of Glu165 due to pK_a decrease in the three relaxation transients probed at 1704 , 1710 , and 1714 cm^{-1} is $320\,000\text{ s}^{-1}$. The fastest rate ($280\,000\text{ s}^{-1}$) in the relaxation transient with the 1721 cm^{-1} probe is not included in the average because of its low amplitude.

Heavy Protein KIE Effects on the Glu165 Deprotonation Kinetics.

The inset in Figure 9 shows the IR T -jump relaxation transients probed at 1665 cm^{-1} of $[\text{U-}^{13}\text{C},\text{-}^{15}\text{N}]\text{- YeTIM/phosphate}$ and $[\text{U-}^{13}\text{C},\text{-}^{15}\text{N}]\text{YeTIM}$, respectively, and their difference. This probe frequency is 2 cm^{-1} higher than the Glu165-protonated carboxyl $^{13}\text{C=O}$ band

(Figure 7), similar in the relative frequency to the 1710 cm^{-1} probe used in the IR T -jump studies on unlabeled YeTIM. The initial intensity drop of the relaxation signal is about 120 mOD in the original spectra, corresponding to $\sim 15\text{ }^{\circ}\text{C}$ temperature increase. The final temperature after the T -jump was $\sim 25\text{ }^{\circ}\text{C}$.

The main transients in Figure 9 show the difference IR T -jump relaxation kinetics between $[\text{U-}^{13}\text{C},\text{-}^{15}\text{N}]\text{YeTIM/phosphate}$ and $[\text{U-}^{13}\text{C},\text{-}^{15}\text{N}]\text{YeTIM}$ at six probe frequencies at 1660 (-3), 1665 ($+2$), 1672 ($+9$), 1677 ($+14$), 1681 ($+18$), and 1702 cm^{-1} . The relaxation transients from other probe frequencies (indicated in Figure 7b) are basically flat (not shown). The numbers in the parenthesis are the relative frequencies to the Glu165 $^{13}\text{C}=\text{O}$ band position (1663 cm^{-1}). These results may be compared to the relaxation curves obtained by the 1704 (-2), 1710 ($+4$), 1714 ($+8$), 1721 ($+15$), 1725 ($+19$), and 1764 cm^{-1} probe frequencies from the unlabeled YeTIM Glu165 $\text{C}=\text{O}$ band (1706 cm^{-1}) (Figure 8). The relaxation curves obtained by probes at 1660 and 1665 cm^{-1} may also be fitted by three exponentials: the faster transition rates are in the $360\,000\text{--}370\,000\text{ s}^{-1}$ range, and two slower transition rates are in the $6000\text{--}9000\text{ s}^{-1}$ and $1100\text{--}1300\text{ s}^{-1}$ range, respectively. The slowest transitions in the $1100\text{--}1300\text{ s}^{-1}$ range have the largest amplitude, and the relaxations are clearly not finished within the 2.3 ms observation time limit. The total relaxation amplitudes are only about 70% of the estimated intensity decrease based on the temperature-dependent static FTIR results (Figure 7). Our results clearly show that the phosphate dissociation-induced deprotonation of Glu165 in $[\text{U-}^{13}\text{C},\text{-}^{15}\text{N}]\text{YeTIM/phosphate}$ is slower than in unlabeled YeTIM/phosphate based on both the rates and amplitudes of the slowest kinetic process. Although we expect a relatively large error in the slowest rates due to unfinished relaxation process in these relaxation transients, we can estimate the KIE due to $^{13}\text{C}/^{15}\text{N}$ labeling of the enzyme (heavy enzyme KIE) = ~ 1.2 for the loop motion-modulated phosphate dissociation process.

The relaxation transients obtained by the 1672 cm^{-1} probe can be curve-fitted by two exponentials: the fast relaxation rate is $350\,000\text{ s}^{-1}$, and the slower rate is 2200 s^{-1} . The average rate for the deprotonation of Glu165 in $[\text{U-}^{13}\text{C},\text{-}^{15}\text{N}]\text{YeTIM/phosphate}$ due to $\text{p}K_{\text{a}}$ decrease in the three relaxation transients probed at 1660 , 1665 , and 1672 cm^{-1} is $360\,000\text{ s}^{-1}$. Although the relaxation transient with the 1677 cm^{-1} probe may also be curve-fitted with two exponentials, its fastest rate ($270\,000\text{ s}^{-1}$) is not included in the average due to the very low amplitude. It is interesting to note here that for the deprotonation of Glu165 due to temperature-induced $\text{p}K_{\text{a}}$ change, we observed an inverse heavy enzyme KIE = 0.89 .

Interpretation of Normal Heavy Enzyme KIEs Observed in Deprotonation Kinetics of Glu165 Carboxyl.

Computational studies based on transition path sampling methods have predicted that for certain enzyme systems, the heavy enzymes may show slowed chemistry at the catalytic site, which may be caused by a slowdown of the reaction coordinate-coupled, fast enzyme motions (promoting vibrations in sub ps or faster time scale) due to protein mass effects. The slowed promoting vibrations decrease the frequency of the system to reach the right protein—substrate coordination for transition-barrier crossing (cf. ref 55). In the earlier heavy enzyme KIE studies on various enzyme systems based on steady-state kinetic methods, more

than half showed slowed chemistry in heavy enzymes and no effects in others (cf. ref 21). Because the steady-state kinetics often reflect the slower enzyme conformational changes, the general interpretation of these heavy enzyme KIEs is that the protein mass increase may also slow (in addition to the promoting vibrations) the global enzyme dynamics such as the catalytic loop motions are related to steady-state kinetics.²¹ The normal heavy enzyme KIE = ~ 1.2 observed for the slower kinetic phase of the Glu165 deprotonation in our IR *T*-jump studies on the TIM/phosphate complex is most likely a reflection of the slowed loop motions in [U-¹³C,-¹⁵N]YeTIM, which is consistent with this general interpretation.

Interpretation of Inverse Heavy Enzyme KIEs Observed in Deprotonation Kinetics of Glu165 Carboxyl.

One of the interesting results in our current IR *T*-jump study is the observation of an inverse heavy enzyme KIE = 0.89 for the tens of μ s deprotonation kinetics of Glu165 carboxyl, while the phosphate is bound to the enzyme. The increased deprotonation rate due to enzyme mass increase seems to be inconsistent with the expected slowdown of enzyme vibrations/motions, but the inverse heavy enzyme KIEs were also observed in previous studies. In a review that summarized 29 reported heavy enzyme KIEs for the chemical step, significant inverse effects were observed in three cases ranging from 0.89 to 0.92.²⁸ Recently, even larger inverse heavy enzyme KIEs (0.71–0.78) were observed in a mutant purine nucleoside phosphorylase (PNP)⁵⁶ and in wt PNP with partial global heavy isotope labeling.⁵⁷ The associated theoretical computations in those studies showed that while the fs bond vibration frequencies become lower in heavy enzymes to slow down the fluctuations of some essential catalytic site contact distances, these contacts may become more frequently optimized in certain specific cases in a longer time period (5 ps in computational studies), and thus may lead to increased probability of forming the transition state.⁵⁷

Implications of Our Results on the Usage of Heavy Enzyme KIEs To Study Enzyme Dynamics and Function Relationship.

The kinetics we observed here are due to protonation/deprotonation of Glu165 carboxyl, which is just a part of the proton abstraction reaction from C1 of DHAP in the first step of the TIM-catalyzed reaction (Scheme 1). Nevertheless, the enzyme structural change due to phosphate binding significantly increases Glu165 pK_a by >3.0 pH units, which correlates to a rate increase for the protonation of Glu165 by more than two orders of magnitude according to a recent NMR study.⁵⁴ This analogy suggests that it is reasonable to discuss our current results in the context of the heavy enzyme isotope effects to understand enzyme dynamics and function relationship.

In a recent review, general trends were summarized based on the results observed in available studies on the heavy enzyme isotope effects: “the absence of ground-state effects may serve to support the hypothesis that enzyme isotope effects on the chemical step are caused by vibrational alteration of the protein, and not electrostatic perturbation of the system. The presence of ground-state effects, on the other hand, may emphasize limitations of the hypothesis but does not preclude its use if transition-state effects can be isolated experimentally.”²⁸ Our phosphate binding studies by fluorescence methods showed no heavy enzyme isotope effects (at least below 250 mM phosphate concentration. Figure S2).

However, our difference FTIR studies showed that there is a small but detectably larger enzyme structural distribution in the heavy YeTIM/phosphate complex near the active site, as indicated by the wider Glu165 carboxyl carbonyl stretch band (compare Figures 3 and 4).

In most heavy enzyme isotope studies, two primary methods have been used to identify the isotope effects on the chemical step as pointed out in a recent review by Schramm and Schwartz: “effects of the mass change in the protein are seen only when the chemical step is isolated from steady-state parameters by presteady-state and substrate trapping (forward commitment) experiments.”²⁷ Typically, the chemical step in these studies is treated as a single step process, and a single chemical turnover rate would be determined with either normal or inverted heavy enzyme KIE, (cf. ref 28) although the values of KIEs may be temperature-dependent and in some cases, even reverse sign with the temperature change.²⁵

Our IR *T*-jump relaxation studies on the Glu165 carbonyl stretch band directly report on the deprotonation kinetics of the Glu165 carboxyl group (the chemical step), which clearly show more than one kinetic process. The slower Glu165 deprotonation kinetics with the largest amplitude is modulated by the loop motion-controlled phosphate release and shows normal heavy enzyme KIE ~ 1.2 . The fast Glu165 deprotonation kinetics, with amplitude 1/6 to 1/5 of the slower phase, is modulated by enzyme motions before phosphate release and shows inverse heavy enzyme KIE ~ 0.89 . Thus, unprecedented detail on the heavy enzyme KIEs may be determined by using this method.

CONCLUSIONS

Our difference FTIR studies on the YeTIM/phosphate complex discovered a C=O stretch band at 1706 cm^{-1} from the Glu165 carboxyl group near neutral pH, indicating that the pK_a of the catalytic base is increased by >3.0 pH units upon phosphate binding, in spite of the fact that the Glu165 carboxyl environment in the complex is still hydrophilic. Previous studies by Richard's lab showed that, besides its function as an anchor for substrate binding, the substrate phosphodianion group binding also activates the enzyme to provide differential energies in the ground state and transition state to reduce the transition barrier.⁴⁷ Our results show that the activation mechanism involves the pK_a elevation of catalytic base Glu165. The slower deprotonation kinetics in the ms time scale is due to phosphate dissociation modulated by the loop motion, which slows down by enzyme mass increase to show a normal heavy enzyme KIE ~ 1.2 . The faster deprotonation kinetics in the tens of the μs time scale is assigned to temperature-induced pK_a decrease while phosphate is still bound, which speeds up in heavy enzyme to show an inverse KIE ~ 0.89 . Our results showed that because of its ability to directly observe the bond breaking/forming kinetics and its μs time resolution, IR *T*-jump spectroscopy can provide high resolution and important detail in the studies of enzyme dynamic and function relationship.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

TIM	triosephosphate isomerase
DHAP	dihydroxyacetone phosphate
GAP	D-glyceraldehyde 3-phosphate
TEV	tobacco etch virus protease
KIE	kinetic isotope effect

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Figure 1.

(a) FTIR spectra of YeTIM/phosphate (8 mM/50 mM, red) and YeTIM (8 mM, blue) obtained in 100 mM Tris, pH 7.5 in D₂O at 20 °C. (b) Difference spectrum between YeTIM/phosphate and YeTIM. (c) Difference spectrum between YeTIM/DHAP (8 mM/10 mM) and YeTIM (8 mM) in 100 mM Tris, 50 mM NaCl, pH 7.5 in D₂O at 20 °C. CaF₂ windows were used with a 25 μ m spacer.

Figure 2.

(a) Difference spectrum between YeTIM/DHAP (8 mM/ 10 mM) and YeTIM (8 mM). (b) Difference spectrum between YeTIM/[¹³C₂]DHAP (8 mM/10 mM) and YeTIM (8 mM). (c) Difference spectrum between YeTIM/phosphate (8 mM/50 mM) and YeTIM (8 mM). All enzyme samples were in 100 mM Tris, 50 mM NaCl, pH 7.5 in D₂O at 20 °C. CaF₂ windows were used with a 25 μ m spacer.

Figure 3.

(a) Active site water molecules in YeTIM/DHAP (1NEY).² (b) Active site water molecules/ion in human TIM (huTIM)/ phosphate (4POC).³⁶ (c) Active site water molecules/ion in the *G. obscuriglobus* TIM (GoTIM)/phosphate complex (4Y96).³⁷

Figure 4. Curve fitting results for the difference spectra between YeTIM/phosphate (8 mM/50 mM) and YeTIM (8 mM) obtained. All enzyme samples were the same as in Figure 2 captions. Voigt line shape was used in the fitting, and the resulting C=O stretch frequencies (and C=O band width) are indicated.

Figure 5.

Curve fitting results for the difference spectra between $[\text{U-}^{13}\text{C}, ^{15}\text{N}] \text{ YeTIM/phosphate}$ (8 mM/50 mM) and $[\text{U-}^{13}\text{C}, ^{15}\text{N}] \text{ YeTIM}$ (8 mM). All enzyme samples were in 100 mM Tris, 50 mM NaCl, pH 7.5 in D_2O at 20 $^\circ\text{C}$. CaF_2 windows were used with a 25 μm spacer. Voigt line shape was used in the fitting, and the resulting C=O stretch frequencies (and C=O band width) are indicated.

Figure 6.

(a) Difference spectra between YeTIM/phosphate (8 mM/ 50 mM) and YeTIM (8 mM) at seven different temperatures from 5 to 35 °C. All enzyme samples were in 100 mM Tris, 50 mM NaCl, Ph 7.5 in D₂O. CaF₂ windows were used with a 25 μ m spacer. (b) FTIR spectrum of YeTIM with indicated IR probe frequencies used in the IR *T*-jump measurements.

Figure 7.

(a) Difference spectra between [U- ^{13}C , ^{15}N]YeTIM/ phosphate (8 mM/50 mM) and [U- ^{13}C , ^{15}N]YeTIM (8 mM) at seven different temperatures from 5 to 35 °C. All enzyme samples were in 100 mM Tris, 50 mM NaCl, pH 7.5 in D_2O . CaF_2 windows were used with a 25 μm spacer. (b) FTIR spectrum of [U- ^{13}C , ^{15}N]- YeTIM with indicated IR probe frequencies used in the IR T -jump measurements.

Figure 8. Difference IR T -jump relaxation transients of YeTIM/ phosphate (3 mM/50 mM) and YeTIM (3 mM) at indicated IR probe frequencies. All enzyme samples were in 100 mM Tris, 50 mM NaCl, pH 7.5 in D_2O . The inset shows the original T -jump relaxation transients of the two samples probed at 1710 cm^{-1} and their difference spectrum. The T -jump was $15\text{ }^\circ\text{C}$, to a final sample temperature of $25\text{ }^\circ\text{C}$. These two enzyme samples were loaded into the split IR cell with $260\text{ }\mu\text{m}$ path length as described in Materials and Methods.

Figure 9.

Difference IR temperature-jump relaxation transients of $[U-^{13}C, ^{15}N]YeTIM/phosphate$ (3 mM/50 mM) and $[U-^{13}C, ^{15}N]YeTIM$ (3 mM) at indicated IR probe frequencies. All enzyme samples were in 100 mM Tris, 50 mM NaCl, pH 7.5 in D_2O . The inset shows the original T -jump relaxation transients of the two samples probed at 1665 cm^{-1} and their difference spectrum. The T -jump was $15\text{ }^{\circ}C$, to a final sample temperature of $25\text{ }^{\circ}C$. These two enzyme samples were loaded into the split IR cell with $260\text{ }\mu m$ path length as described in Materials and Methods.

Scheme 1.

Table 1.Observed Relaxation Rates by IR T -Jump

probe (shift) cm^{-1}	rates $k \text{ s}^{-1}$		
TIM/Pi	rate 1	rate 2	rate 3
1704 (−2)	301	9.1	1.60
1710 (+4)	35	7.6	1.26
1714 (+8)	302	2.4 ^a	
average	321 (±30)		1.43 (±0.3)
[U- ¹³ C, ¹⁵ N] TIM/Pi			
1660 (−3)	369	8.6	1.24
1665 (+2)	366	5.8	1.11
1672 (+9)	352	2.2 ^a	
average	362 (±30)		1.18 (±0.3)
heavy E KIE	0.89		1.2

^a Because of low amplitude in relaxation transients obtained with 1714 or 1672 cm^{-1} probe, rate 2 and 3 are likely merged to show this apparent value. Probe (shift): IR probe frequencies in IR T -jump (frequency shift relative to the center C=O stretch frequency of the Glu165 carboxyl); all rates were determined from observed transients by curve fitting with 3 exponentials. The standard deviations for the fitted rates are typically 3% or less.