Published in final edited form as:

J Mol Biol. 2010 March 19; 397(1): 233–248. doi:10.1016/j.jmb.2009.10.043.

Triosephosphate Isomerase: ^{15}N and ^{13}C Chemical Shift Assignments and Conformational Change upon Ligand Binding by Magic-Angle Spinning Solid-State NMR Spectroscopy

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Abstract

Microcrystalline uniformly ^{13}C , ^{15}N -enriched yeast triosephosphate isomerase (TIM) is sequentially assigned by high-resolution solid-state NMR (SSNMR). Assignments are based on intrareidue and interresidue correlations, using dipolar polarization transfer methods, and guided by solution NMR assignments of the same protein. We obtained information on most of the active-site residues involved in chemistry, including some that were not reported in a previous solution NMR study, such as the side-chain carbons of His95. Chemical shift differences comparing the microcrystalline environment to the aqueous environment appear to be mainly due to crystal packing interactions. Site-specific perturbations of the enzyme's chemical shifts upon ligand binding are studied by SSNMR for the first time. These changes monitor proteinwide conformational adjustment upon ligand binding, including many of the sites probed by solution NMR and X-ray studies. Changes in Gln119, Ala163, and Gly210 were observed in our SSNMR studies, but were not reported in solution NMR studies (chicken or yeast). These studies identify a number of new sites with particularly clear markers for ligand binding, paving the way for future studies of triosephosphate isomerase dynamics and mechanism.

Keywords

triosephosphate isomerase; solid-state NMR; sequential assignment; crystal packing; conformational change

Introduction

Triosephosphate isomerase (TIM) catalyzes the central reaction in the glycolytic pathway—the reversible interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate—while suppressing the production of methyl glyoxal by phosphate elimination side reaction.¹ Studies of the mechanism support the existence of a chemical intermediate, the enediol(ate) of DHAP. As a catalyst, TIM has often been said to have achieved

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.10.043

evolutionary perfection, and it has been extensively characterized with numerous structural,² mechanistic,³ and kinetic⁴⁻⁶ studies.

A dramatic conformational change in TIM induces proton redistribution in the enzyme's active site. The ligand-induced structural change was first observed in chicken TIM;⁴ subsequently, key players involved in catalysis were extensively explored by X-ray crystallography⁵⁻⁸ and site-specific mutations.⁵⁻⁹ In high-resolution crystal structures of unligated TIM, loop 6 was observed in the so-called open form;^{10,11} when the protein is ligated either by the substrate or by substrate analogues, loop 6 is usually closed¹² (although some exceptions have been found¹³⁻¹⁶). Comparison of the open and closed conformations of chicken, yeast, and trypanosomal TIM structures indicates that the various open forms are identical with each other, and that the closed forms of yeast and trypanosomal TIMs are essentially indistinguishable from each other.¹⁷ Closure of loop 6 on the active site organizes the side chains of Asn10, Lys12, His95, Ser96, Glu97, Glu165, and Ser211, and the backbones of Gly232, Gly233, and Gly171 to interact with and surround the substrate, thus accelerating the reaction by 10 orders of magnitude. Simultaneously, its closed conformation protects the substrate from contact with bulk water, thereby presumably preventing the deleterious elimination of phosphate from the intermediate.⁵

High-resolution NMR is a very sensitive tool used for detecting conformational dynamics at the atomic level. Isotropic chemical shift is sensitive to local structure, especially torsional states of the chain and side chains, and also to electrostatics, solvent exposure, and ligand binding. Previous solution NMR studies have reported most backbone and side-chain assignments for unligated and D-glycerol 3-phosphate (D-G3P) (or 2-phosphoglycolate)-ligated yeast¹⁸ and chicken¹⁹ TIMs. In yeast TIM, G3P-induced chemical shift perturbations were observed for Asn10, His95, Ser100, Glu129-Glu133, Val167-Ala186, Asn213, and Leu230-Leu126 residues that either are near the ligand or have direct contact with the ligand, with some key residues, including Glu165, Gly210, Ser211, and Ala212, missing from the data set. 2-Phosphoglycolate binding to chicken TIM also shows significant chemical shift perturbations on the residues that are close to the ligand or interact with the ligand. These chemical shift perturbations offered an opportunity for studying the dynamics of binding and conformational exchange in a site-specific fashion.

Solid-state NMR (SSNMR) is a powerful tool that complements other biophysical methods in studying the structure, mechanism, and dynamics of bio-molecules. Because of the ability to vary temperature, the Michaelis complex can be stabilized for SSNMR studies, allowing access to issues of great chemical interest. Also, because of the ability to vary the temperature broadly, SSNMR methods might have access to key catalytic sites, including ones that are invisible in solution NMR studies due to extensive line broadening from chemical exchange. The microcrystalline samples employed in SSNMR are very similar to the crystals used in X-ray crystallography, allowing for direct comparisons between the X-ray structure and the SSNMR data. For all these reasons, SSNMR spectroscopy has the potential to reveal novel mechanistic aspects.

To date, SSNMR studies of TIM have offered a limited view of the active site because they required site-specific isotopic enrichment of the unique sites. For example, to probe loop

dynamics, we used isotopic enrichment of a unique tryptophan residue in a fully active mutant of the enzyme, namely, Trp168.²⁰ SSNMR studies of many of the key active-site catalytic players have been limited by our inability to carry out site-specific enrichment. For a more complete access to dynamic and chemical information, the more direct approach is to assign the signals for an extensively or uniformly isotopically enriched sample. Assignments by SSNMR have been greatly facilitated by the development of magic-angle spinning²¹ and sequential assignment protocols;^{22,23} for proteins as large as TIM, however, chemical shift assignments are still, by no means, routine. In this report, we present site-specific SSNMR sequential assignments of uniformly ¹³C,¹⁵N-enriched microcrystalline yeast TIM, as guided by the published solution NMR assignment.¹⁸ We compare chemical shifts in solution *versus* chemical shifts in microcrystalline form. Subsequently, the substrate analogue D-G3P was cocrystallized with the enzyme, and chemical shift perturbations were detected. These observations provide a number of markers for further site-specific dynamic studies and active-site chemical players, and thereby open the possibility of unveiling the TIM catalytic mechanism.

Results and Discussion

Sequential assignment strategy and chemical shift assignments

Yeast TIM, a 247-residue protein with a molecular mass of 26 kDa, is one of the largest systems studied by SSNMR. Most resonances for backbone N, C^α, and CO, and for side-chain C^β have been assigned by solution NMR (BioMagResBank 7216),¹⁸ which were used to help identify SSNMR assignments. Backbone and side-chain sequential SSNMR assignments (see Supplementary Table 1) were based on five spectra: two-dimensional (2D) homonuclear ¹³C–¹³C spectrum,²⁴ 2D heteronuclear NCA and NCO spectra,^{25,26} and three-dimensional (3D) NCACX and NCOCX spectra combining the two experiments for the purpose of sequential assignment, as we have done previously.²² NCACX, NCOCX, NCA, and NCO spectra contained backbone correlations. The NCACX, NCOCX, and ¹³C–¹³C spectra were used for side-chain assignments (for details on pulse sequences, see Materials and Methods).

Our sequential assignment strategy was similar to our previously published protocols for “backbone walks.”^{22,23,27} In addition, solution assignments were displayed with the program CARA (Computer-Aided Resonance Assignment),²⁸ which allowed convenient comparisons of resonance correlations for SSNMR and solution NMR, and thereby gave us leading hypotheses for assignments in many cases. For regions with clear solid solution perturbations or that are highly congested, a spectra combination of 2D and 3D SSNMR data was used. For example, for the resonances in Thr60, correlations of C^α C^β, C^α Cγ², C^β Cγ², C^αCO, and C^β CO in ¹³C–¹³C 2D; NC^α C^β and NC^α CO in NCACX 3D; and Thr60N-Val59CO-Val59C^α, Val61N-Thr60CO-Thr60C^α, and Val61N-Thr60CO-Thr60C^β in NCOCX 3D were all found and used to make the resonance assignments of the backbone and side-chain atoms of Thr60. Consequently, a number of new resonance assignments not reported by solution NMR were discovered. Figure 1 summarizes these new resonance assignments in the solid state, as well as all the interresidue and intrasidue correlations

found. In the 2D homonuclear and heteronuclear spectra, the average linewidth of resonance peaks is 0.3 ppm in the carbon dimension and 0.5 ppm in the nitrogen dimension.

Comparison of solid-state and solution NMR assignments

There has been great interest^{29–31} in studying the differences and similarities of chemical shifts between the solution state and the crystalline state. Cole and Torchia were the first to report a close agreement in nitrogen chemical shifts between solution-state staphylococcal nuclease and crystalline staphylococcal nuclease.³¹ In another study, Detken *et al.* reported a remarkable agreement in backbone and aliphatic side-chain assignments for a 10-residue molecule, antamanide.³⁰ The first nearly fully assigned protein by SSNMR, the SH3 domain of α -spectrin,³² exhibited isotropic chemical shifts from solution NMR and SSNMR that were in overall agreement, with the exception of a few sites.³³ Spectra of the *Bacillus subtilis* protein Crh in solution³⁴ and in solid state³⁵ exhibited large chemical shift differences that were attributed to the dimerization of Crh.³⁵ Ubiquitin was also assigned by solution NMR³⁶ and SSNMR.^{22,23} The two samples differed in pH and buffer conditions, and residues with the greatest chemical shift deviations were generally polar or solvent exposed. Crystal packing interactions are also thought to affect chemical shifts.^{22,23} Franks *et al.* assigned GB1 by SSNMR,²⁹ and crystal packing interactions were proposed to influence rotameric states and intermolecular interactions between β 2 and β 3 strands, accounting for chemical shift differences with the solution state. Most reports to date show general agreement between solution state and solid state, where perturbations are generally attributed to differences in sample preparation, solvent exposure, or crystal packing interactions.

Figure 2 plots the crystal-*versus*-solution differences in chemical shifts for TIM, which are defined as $\Delta\delta^i = \delta_{\text{solid}}^i - \delta_{\text{solution}}^i$ ($i = {}^{13}\text{C}^\alpha$, ${}^{13}\text{C}^\beta$, ${}^{13}\text{C}$, and ${}^{15}\text{N}$). The mean of the chemical shift differences is near zero, and the RMSD is about 0.5 ppm, demonstrating a very good overall agreement between solution-state and solid-state shifts. Outliers were identified by constructing histograms of chemical shift differences for C^α , C^β , CO, and N (Fig. 3). The histograms contain 166 resonances for C^α , 144 resonances for C^β , 137 resonances for CO, and 123 resonances for N. Histograms were fitted with Gaussian curves, and outliers were defined as residues that were outside $\pm 1.96\sigma$ from the mean (i.e., outside of the 95% confidence interval). All outliers are listed in Table 1.

Any difference could be due to the solvent conditions, in that the conditions are somewhat different. In this regard, we were confident that the enzyme we studied here was fully competent under the experimental conditions, both in solution (the mother liquor) and in the crystalline (or precipitated) form. Prior solution NMR studies were carried out at pH 5.9 in a sodium acetate buffer,¹⁸ whereas the SSNMR samples studied here were prepared at pH 6.8 in a Tris buffer [with polyethylene glycol (PEG) precipitation]. The difference in pH could produce changes in local charge distributions, consistent with the fact that TIM has a slightly different activity between these two pH values.³⁷ Furthermore, polar surface residues may form intermolecular contacts not observed in monodispersed TIM in solution, leading to chemical shift changes in these residues.

Chemical shift differences comparing solution state and solid state could also be due to crystal contacts. Based on the Protein Data Bank (PDB) structure for the wild-type yeast TIM (PDB ID 1I45³⁸), the crystal packing structure was mapped with the program O.³⁹ We chose the 1I45 structure because it matches the crystallization conditions used in this work.³⁸ The crystal contacts were calculated with ACCEPT-NMR (*Automated Crystal Contact Extrapolation/Prediction Toolkit for NMR*).⁴⁰ They were defined as all residues with at least one atom within 5 Å of an atom of a residue in an adjacent molecule in the crystal (see Supplementary Table 3). Indeed, many of the perturbed chemical shifts are at or near crystal contacts. Among the residues that have chemical shift perturbations between solution NMR and SSNMR assignments (Fig. 4), Thr4 in the N-terminus, Asn28, Thr29, Ser31, and Asn35 in the small loop after helix 1 are all involved in direct crystal contacts for chain B in the microcrystalline condition. Thr60 is located in a small β -strand (sheet 3) in the core of the bundle, with its side-chain exposed to the solvent. Although it does not have direct crystal contacts with other subunits, neighboring residues Lys55 and Lys56 are involved in crystal contacts.

Active-site residues detected by SSNMR, not by solution NMR

The three residues Lys12, His95, and Glu165 are near the active site and are known to be critical for the chemistry of proton transfer in the substrate (Fig. 5).^{10,41} Remarkably, two of these residues, Lys12 and His95, have been observed and assigned in our SSNMR spectra. The anionic phosphate moiety of the substrate is stabilized by hydrogen bonds to Gly209, Gly210, Ser211, Gly171, Gly232, and Gly233. These are all identified in our studies; this is noteworthy since residues Gly209, Gly210, and Gly233 are absent from the solution assignments.

Although our study reports many new resonance assignments, some residues are absent—most notably Glu165, which is of particular interest for studying the mechanism and is missing in both the solution data set and the solid-state data set. This residue is responsible for the initiation and presumably also the termination of TIM catalysis, and it is involved in one of the postulated proton transfer pathways mentioned before.⁴² Either the Glu165 cross-peaks are occluded by other peaks with similar chemical shifts or the peak intensities are attenuated by motion. Multidimensional spectra at higher resolution, selective labeling schemes, lower temperature studies, or a tighter inhibitor are the different avenues being pursued to further characterize this important site.

Conformational changes in ligand binding as detected by SSNMR

The study of TIM with the native substrate is complicated by the rapid elimination of phosphate, which produces a highly reactive methylglyoxal byproduct at temperatures above 0 °C.⁴³ Therefore, we have characterized the binding of a substrate analogue D-G3P, which has a structure and a binding affinity similar to those of the native substrate DHAP: The steady-state parameters for baker's yeast TIM expressed in *Escherichia coli* at 30 °C are $K_i = 1.4 \pm 0.3$ mM for D-G3P and $K_m = 1.4 \pm 0.1$ mM for the substrate.⁴⁴ The TIM–G3P complex is also much more stable than the TIM–DHAP complex at temperatures above 0 °C.

Partial assignment of NMR signals for the G3P-ligated crystalline enzyme was achieved with homonuclear ^{13}C - ^{13}C 2D spectra and heteronuclear ^{15}N - ^{13}C CA, ^{15}N - ^{13}C CO 2D spectra (see Supplementary Table 2). Figure 6 shows an overlay of the homonuclear ^{13}C - ^{13}C 2D spectra of apo TIM and ligated TIM. The differences in chemical shifts between the bound form and the free form are plotted in histograms for C^α , C^β , CO, and N, and were fitted using a Gaussian function. We defined outliers as all residues that lay outside of the 95% confidence interval ($\pm 1.96\sigma$) from the mean. All residues that are involved in chemical shift perturbations upon binding are mapped in Fig. 7a. They include Gly9, Asn14, Thr29, His95, Gln119, Gly128, Glu129, Ala163, Trp168, Thr172, Gly173, Ala176, Thr177, Ile184, Gly210, Ser211, Gly228, and Phe229. Figure 7b shows the residues with chemical shift perturbations by solution NMR.¹⁸ Consistent with solution NMR data, our assignments show that the residues closest to the ligand have large chemical shift changes upon ligand binding.^{18,19} Their roles are discussed in more detail below.

The chemical reaction is initiated by abstraction of the pro-R proton from C1 of DHAP by Glu165, the catalytic base.⁴⁵ In the crystal structures, the conformational change upon ligand binding reorients the backbone and side-chain torsion angles of Glu165 from ($\phi=-110.3$, $\psi=112.5$, $\chi_1=-75.5$) to ($\phi=-129.2$, $\psi=99.7$, $\chi_1=-47.4$), shifting the side chain by 2–3 Å. In this process, Glu165 rotates to mediate proton transfer between C1 and C2 carbons of the ligand. We were unable to confidently identify the catalytic base Glu165 in either the ligated state or the unligated state of the enzyme, which was also the case for prior solution NMR studies. Previously, 14 of the 17 total glutamates were partially assigned (N, CO, C^α , and C^β) in solution, and we report here the backbone assignment of eight (residues 22, 34, 37, 77, 104, 129, 203, and 239). The missing assignments of the C^γ and C^δ atoms of four residues (residues 34, 239, 104, and 203) were assigned in the solid state. Although shifts are observed with peaks in the C^γ - C^δ region of our ^{13}C - ^{13}C 2D spectra, their site-specific assignment will require spectra with better resolution and signal-to-noise ratio.

The electrophilic side-chain of neutral His95 polarizes the carbonyl group (O2) of the substrate^{46,47} and thereby is a crucial residue for facilitating the reaction. His95 is positioned to interact with the O1 and O2 groups, and thereby to stabilize the transition state and the intermediate.⁴⁸ The protonation states of His95 and the pathways of proton transfer remain topics of debate in the literature, and direct NMR measurements will be useful in settling these issues. The backbone of His95 was detected and assigned in the solution NMR studies; in addition, we have detected $\text{C}^{\delta 2}$, adjacent to $\text{N}^{\epsilon 2}$,^{48,49} for which we observed a change in chemical shift upon substrate binding. In one model, His95 donates a proton from $\text{N}^{\epsilon 2}$ to the substrate O2 atom to form an enediol(ate) intermediate and subsequently abstracts a second proton from the substrate O1 atom.^{50–54} In another model, Glu165 is responsible for shuttling this proton.^{42,51,52} In a third model, a direct internal proton transfer from O1 to O2 forms the intermediate.^{45,50,53} Mutations of His95 to Gln or Asn reduced $k_{\text{cat}}/K_{\text{m}}$ (DHAP as substrate) to 0.006 and 0.003, respectively, compared to the wild type.⁴⁶ Lodi and Knowles reported $^{13}\text{C}^\gamma$ chemical shift changes in His95, His103, and His185 under different pH and binding conditions, using solution NMR and chemical titrations.⁴⁸ Both His95 and His185 had unchanged chemical shifts over the range from pH 4.3 to pH 9.5. His95 had different C^γ shifts, depending on the ligand, and the C^γ resonance of His185 consistently shifted in the same direction. The His103 C^γ resonance shifted downfield with an increase in pH, but it

remained unperturbed upon ligand binding. All three of these histidine atoms were identified in this work, and their C β and C γ resonances remained unchanged upon ligand binding (discussed in a later section). The fact that the solution NMR study reports a change in the His185 C γ resonance—and we do not—could be due to a different choice of ligand, but is a point that requires further investigation. On the other hand, in our data, the C δ^2 resonance of His95 moves downfield, from 115.8 ppm (apo) to 117.4 ppm (bound), upon ligand binding. This change in chemical shift at C δ^2 , next to the N ϵ^2 donor nitrogen, is unambiguous evidence that His95 is an electrophile in this context. This assignment can be used to further investigate the catalytic mechanism of TIM with the native substrate.

A number of other active-site residues would be expected to shift upon binding, including those that are indirect reporters on the motions of Glu165, the (spectroscopically elusive) catalytic base. The side-chain carboxylate moves from an aqueous environment to a hydrophobic environment enclosed by residues Ala163, Ile170, Gly209, and Leu230. The distance between the Ala163 C β atom and the Glu165 carboxylate carbon decreases from 6.2 Å in the unbound state to 5 Å in the bound state. The observed shift in Ala163 C β (−0.8 ppm) in this work reports the approach of the negatively charged catalytic base (−COO $^-$). Ala163 shows changes in its C α , C β , CO, and N chemical shifts, which disappear upon binding in the yeast solution NMR study. The chicken solution NMR study only reports the C β and N shifts, which both remain unchanged upon binding. Ala163 is located outside of the active-site pocket, and it does not form direct hydrogen bonds to the substrate. In both the unbound structure (1I45¹¹) and the substrate-bound structure (1NEY¹²), Ala163 is in close contact with residues in sheet 6 (residues 161, 162, 164, and 165): it is flanked by sheet 5 (residues 124–127) on one side and by Leu130 and Leu207 on the other side.

In addition, a number of ligands for the phosphate “anchor” the substrate and were also observed to shift, as expected, upon ligand binding. The amide nitrogen of Gly233 is directly hydrogen bonded to the phosphate oxygens in the substrate. Ser211, whose backbone binds the phosphate oxygen of the substrate, is not assigned in the solution studies, but is observed here. The amide nitrogens of Gly232 and Gly233 are directly hydrogen bonded to the phosphate oxygens in the substrate. We have assigned the C α and CO atoms of Gly232 in both ligated and unligated TIMs, and a change in chemical shift was observed for both atoms. The C α –CO cross-peak (for Gly233) appears in a congested region of the spectrum; therefore, we leave our observation of the shift in Gly232 out of the current discussion. Gly210, located in the YGG motif in loop 7 (Tyr208–Gly209–Gly210–Ser211), is another active player in the restructuring loop 7 and in its interactions with loop 6 during binding. Binding induces a rotation of 90° for the peptide plane between Gly209 and Gly210, and a rotation of almost 180° for the peptide plane between Gly210 and Ser211.¹⁵ A hydrogen bond between the Gly210 amide proton and the Tyr208 hydroxyl group forms upon binding of G3P¹⁵ (in yellow in Fig. 7c). In the ligated enzyme, Gly210 reorients to form a hydrogen bond with a water molecule, which in turn forms a hydrogen bond to the phosphate oxygen of the substrate. In this study, we observed changes in the isotropic shift not seen previously in solution NMR. Solution NMR studies did not identify the peaks associated with this residue, possibly because they were broadened by chemical exchange. SHIFTX (Fig. 7c) predicts a chemical shift perturbation for this residue upon ligand binding.

In addition, there are a number of broader reporters of the well-studied conformational exchange of the active-site loops. Upon ligand binding, loop 6 clamps down over the active site, and loop 5 tilts slightly in response to the change in the hydrogen bond partner in loop 6. There is also a dramatic movement of loop 7, helping to position Glu165 into the active site. Hydrogen bonds between the C-terminus of loop 6 and the YGGs motif in loop 7, Gly173-Ser211, and Ala176-Tyr208 are loosened to initiate the loop opening. Ser211 positions its backbone amide to bind the phosphate oxygens of the ligand. In the unligated enzyme, the amide nitrogen of Gly210 points towards the aromatic ring of Tyr208.¹⁵ Ala163 in sheet 6, Ile184 in helix 6, Asn14 in loop 1, Thr29 in helix 1, Gly9 in sheet 1, and Gln119 in the loop after helix 4 all exhibit corresponding structural adjustments with the conformational change. Figure 7c shows additional residues involved in conformational changes, as observed by SSNMR (yeast TIM), solution NMR (yeast TIM), solution NMR (chicken TIM), and X-ray crystallography (yeast TIM), and as predicted by SHIFTX.⁵⁵ Several residues show changes upon binding in these SSNMR studies that were not observed by the other methods. For example, Gln119 has a 0.6-ppm shift in C β resonance upon ligand binding, and it remains unchanged in the chicken TIM solution NMR data.

The resonance peaks that are shifted in the ¹³C-¹³C 2D spectra upon binding are potentially markers for future dynamic or titration studies. Good markers should be well resolved and sharp in both ligated and unligated forms. The cross-peaks for Ala163 C β -C α , Gly210 C α -CO, Thr172 C β -C α , and Thr177 C β -C α stand out as candidates. Furthermore, assigned residues with no isotropic shift change, but with a change in orientation (such as Ala169), are good candidates as probes for dynamics by SSNMR.

Crystal packing in the Michaelis complex

The residues involved in crystal contacts for the substrate-bound structure were calculated with ACCEPT-NMR (Supplementary Table 4). Our sample was prepared by cocrystallization and under conditions nearly identical with those used for the preparation of the 1NEY¹² crystal; therefore, we assume that the crystal lattice and packing in our sample will be similar.

Figure 8 illustrates a comparison of crystal packing patterns between apo enzymes and ligated enzymes. The residues involved in crystal packing are mostly the same between the two samples, although more crystal packing interactions are observed for the ligated structure. Both subunits of loop 6 participate in packing interactions for the ligated and unligated proteins; the hydrogen bonding partners, however, are different for this loop in both forms. The open and closed states of loop 6 are stabilized by crystal packing interactions in both structures. From all of the changes in chemical shift observed upon ligand binding, only one appears to be associated with reorganization of the crystal packing. The loop after helix 4, with residues His103, Lys107, Lys114, and Gly118, gets involved in crystal packing interactions only after the ligand has been bound. Furthermore, although Gln119 (the direct neighbor of Gly118 on that loop) does not have a direct crystal contact, the packing force applied on its neighbor might affect its secondary structures, making this loop slightly distorted. This crystal packing distortion can result in a change in local

environment, and it may only happen in the crystalline samples, explaining why Gln119 was observed to have isotropic shifts in our SSNMR studies, but not in solution.

Conclusions

We have presented the ^{15}N and ^{13}C chemical shift assignments for microcrystalline TIM by SSNMR. We observe an overall agreement with prior solution assignments. Most active-site residues are assigned in this work, including a few that were not observed by solution NMR. Of particular interest, the His95 side-chain ($\text{C}^{\delta 1}$) assignment will be invaluable in ascertaining the role of His95 in proton transfer.

Some of the observed chemical shift perturbations, as compared to the solution NMR assignments, are related to crystal packing interactions. There are 42 residues that are within 5 Å of an adjacent molecule in the lattice, of which 5 residues show shifts above the cutoff of 0.4 ppm for C^{α} and C^{β} , the cutoff of 0.5 ppm for CO, and the cutoff of 1.1 ppm for N. If we decrease this cutoff to 4 Å, 3 residues would show shifts out of 29 residues that are involved in crystal contacts; if the cutoff is set to 3 Å, 1 residue (Thr29) would show shifts out of 1 residue (Thr29) that is involved in crystal contacts. Of the eight shifts that are above the cutoff of 0.4 ppm for C^{α} and C^{β} , the cutoff of 0.5 ppm for CO, and the cutoff of 1.1 ppm for N, six are on or near crystal contacts (cutoff, 5 Å). Therefore, large changes in chemical shift might be useful in locating crystal contact sites, although sites involved in crystal contacts may not, conversely, have particularly large chemical shifts.

For the first time, we report site-specific SSNMR chemical shift perturbations upon ligand binding. The chemical shift perturbations between ligated TIM and unligated TIM show conformational changes throughout the protein. Upon binding, we found 17 residues with large changes in chemical shift. Of these, 5 residues (His95, Thr172, Gly173, Gly210, and Ser211) are in direct contact with the ligand, 14 residues (Gly9, Asn14, Thr29, His95, Gly128, Glu129, Thr172, Gly173, Ala176, Thr177, Ile184, Ser211, Gly228, and Fhe229) have structural changes observed by either X-ray or solution NMR studies, and 2 residues (Thr29 and Thr172) are located at crystal packing sites that have changed. There are also residues that are in ligand contact but remain unshifted upon binding, such as Ala169. Future structural and dynamic work should be enabled by our discovery herein of the many active-site residues with well-resolved markers for binding, including His95, Ala163, Thr172, Thr177, and Gly210.

Materials and Methods

Materials

All reagents used were purchased from Sigma-Aldrich, with the exception of glycerol-3-phosphate dehydrogenase, which was purchased from Roche Diagnostics Corporation (Germany). Isotopically enriched chemicals, including D-glucose ($\text{U-}^{13}\text{C}_6$; 99%; CIL no. CLM-1396), ammonium chloride (^{15}N ; 99%; CIL no. NLM-467), and 1,3- ^{13}C glycerol, were purchased from Cambridge Isotope Laboratories.

Expression and purification of yeast wild-type TIM

Uniformly ^{13}C , ^{15}N -enriched wild-type recombinant *Saccharomyces cerevisiae* TIM was prepared by overexpression in *E. coli* and purified, as described elsewhere.⁵⁶

SSNMR samples

For the apo TIM sample, the enzyme was concentrated to 200 mg/ml in 50 mM Tris-HCl, 50 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (pH 6.8) at 4 °C and 40% (wt/vol) PEG (average molecular weight, 4000). The buffer was gently added to the protein solution in a sealed pipet tip to reach a final concentration of 15%, and the solution was stored overnight at 4 °C to form crystals. At lower ionic strengths [25 mM Tris-HCl, 25 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (pH 6.8), at 4 °C], amorphous precipitation occurs.

For the ligated samples, the ligand was added in a 5:1 ligand/enzyme ratio. Ligand saturation was monitored by measuring inhibition of enzyme activity. The ligand and the precipitant were dissolved in 10 mM Tris-HCl (pH 6.8) at 4 °C to minimize ionic strength. Addition of the magnesium salt of the ligand did not change the solution pH. After an hour of incubation at 4 °C, 40% (wt/vol) PEG 4000 was added to reach the final concentration of 15%. After an overnight incubation at 4 °C, crystals were visible. A typical 4-mm SSNMR Varian rotor contained 20 mg of hydrated microcrystalline protein, approximately 35 μl of the sample.

Activity assays

Activity assays were performed as previously reported (Fig. 9).⁵⁶

SSNMR spectroscopy experimental conditions

Resonance assignments were determined from data collected on either a Bruker Avance DRX-750 spectrometer or a Bruker Avance DRX-900 spectrometer (New York Structural Biology Center). Conformational change experiments were acquired on a Varian Infinity 600-MHz spectrometer.

Two-dimensional ^{13}C - ^{13}C homonuclear correlation spectra were acquired using DARR (*D*ipolar-*A*ssisted *R*otational *R*esonance) mixing.²⁴ Two-dimensional ^{15}N - ^{13}C heteronuclear NCA and NCO spectra were acquired by selective double cross-polarization.^{25,26} Three-dimensional NCACX and NCOCX spectra were acquired using these two transfer methods sequentially: double cross-polarization-DARR.²²

Data were processed and analyzed with NMRPipe⁵⁷ and SPARKY.⁵⁸ Spectra were processed with linear prediction,⁵⁹⁻⁶² and baselines were corrected using a polynomial function.⁶³ Apodization was achieved using either a cosine squared function,⁶⁴ a Gaussian function,⁶⁵ or a mixed Lorentzian-Gaussian function.

Referencing of the ^{13}C Larmor frequency relative to 2,2-dimethyl-2-silapentane-5-sulfonic acid was achieved using an external standard: the ^{13}C adamantane methylene peak at 40.26 ppm.⁶⁶ The nitrogen dimension was calibrated by indirect referencing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ivan Sergeyev for his program ACCEPT-NMR (<http://mcdermott.chem/software/>) used for crystal contact calculations. We thank previous and current McDermott group members for technical assistance. A.E.M. is a member of the New York Structural Biology Center. The New York Structural Biology Center is a STAR center supported by the New York State Office of Science, Technology, and Academic Research. This work was supported by funds from the National Institutes of Health.

Abbreviations used

TIM	triosephosphate isomerase
SSNMR	solid-state NMR
DHAP	dihydroxyacetone phosphate
G3P	DL-glycerol 3-phosphate
D-G3P	D-glycerol 3-phosphate
2D	two-dimensional
3D	three-dimensional
PEG	polyethylene glycol
PDB	Protein Data Bank

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Fig. 1.

Interresidue and intraresidue correlations employed in SSNMR assignments. Residues in red have intraresidue correlations available; residues in blue have interresidue correlations available; residues in orange have intraresidue or interresidue correlations independent of solution NMR available; residues in green are the ones unassigned in solution NMR.

Fig. 2.

Chemical shift differences comparing chemical shifts for yeast TIM in solution *versus* chemical shifts for yeast TIM in the microcrystalline solid state. The mean difference (average of solid-solution) and the standard deviation in this difference (σ) are given for each atom type: 0.1 ± 0.2 ppm for $C\alpha$, 0.1 ± 0.2 ppm for $C\beta$, -0.2 ± 0.2 ppm for CO , and 0.0 ± 0.4 ppm for N . In general, a close agreement between solution NMR and SSNMR assignments was observed. A handful of sites with chemical shift perturbations at least 1.96σ away from the mean (red lines) are defined by the blue lines: Gly232 for $C\alpha$; Thr29, Ser31, and Thr60 for $C\beta$; Asn28 and Asn35 for CO ; and Thr4, Thr60, and Thr128 for N .

Fig. 3.

Histograms are presented for chemical shift differences in the solid-state and solution NMR assignments for C^α , C^β , CO, and N. They contain 166 observations for C^α , 144 observations for C^β , 137 observations for CO, and 123 observations for N. Continuous lines are the best-fitting Gaussian curves. Best-fit values of expected value and standard deviation were found to be 0.15 ± 0.02 and 0.26 ± 0.07 for C^α , 0.11 ± 0.05 and 0.28 ± 0.07 for C^β , -0.16 ± 0.02 and 0.18 ± 0.03 for CO, and 0.03 ± 0.03 and 0.33 ± 0.03 for N. Broken lines indicate sites 1.96σ away from the mean. Together with Fig. 2, the histograms show an overall agreement between solution NMR and SSNMR assignments, and point out the outliers: Gly232 for C^α ; Thr29, Ser31, and Thr60 for C^β ; Asn28 and Asn35 for CO; and Thr4, Thr60, and Thr128 for N.

Fig. 4. The relation between crystal packing and chemical shift perturbations between solution NMR and SSNMR assignments. The residues that have crystal contacts (red) are mapped onto a dimer of yeast TIM (PDB ID 1I45¹). The ones that have chemical shift perturbations *and* are also on or near crystal contacts (Thr4, Asn28, Thr29, Ser31, Asn35, and Thr60) are labeled in purple; the ones that have chemical shift perturbations but are *not* in crystal contacts (Gly128 and Gly232) are labeled in green. Most perturbed residues (six of eight) are on or near crystal contacts.

Fig. 5.
The active site of TIM bound with the substrate (PDB ID 1NEY²). Residues labeled in red are the ones observed in SSNMR but not in solution NMR; residues labeled in blue are the ones observed in both; residues labeled in gray are not observed in either.

Fig. 6. The overlay of the ^{13}C - ^{13}C 2D spectra of apo TIM (red) and D-G3P ligated TIM (green). Insets: Carbon correlations for the Thr C^β - C^α , Gly C^α -CO, and Ala C^β - C^α regions. The spectrum of apo TIM was acquired on a Bruker Avance DRX-750 spectrometer; the spectrum of D-G3P-bound TIM was acquired on a Varian Infinity 600-MHz spectrometer.

Fig. 7.

All residues involved in chemical shift perturbation upon ligand binding of G3P detected by (a) SSNMR and (b) solution NMR. (a) SSNMR markers that are well resolved and in contact with substrate include Ala163, Thr172, Thr177, and Gly210. They are labeled in blue, and the insets are their corresponding peaks in the overlay CC spectra of apo TIM (red) and D-G3P bound TIM (green). (b) The residues from solution NMR are the ones with backbone nitrogen shifted no less than 0.5 ppm upon binding of G3P.³ The schematic structure of the TIM monomer is the yeast TIM complexed with the substrate DHAP (PDB ID 1NEY¹). (c) Residues involved in conformational change detected by SSNMR in yeast TIM (red), by solution NMR in yeast TIM³ (blue), and by solution NMR in chicken TIM⁴ (pink), and predicted by SHIFTX⁵ (green; PDB IDs 1I45 and 1NEY), mapped onto the primary sequence of yeast TIM. The residues that are not observed in both apo and ligated TIMs by NMR are coded in black. The active-site residues observed by X-ray are coded in yellow. The chicken TIM primary sequence is slightly different from that of the yeast TIM; therefore, mapping is based on their correlation.⁶ Most (15 of 18) shifts in SSNMR data are near or at substrate contacts (yellow). The rest (Thr29, Gln119, and Ile184) curiously are all on or near the regions where solution NMR also shows shifts.

Fig. 8. Residues involved in crystal packing color-coded onto the apo TIM structure 1I45.¹ The ones in red are for the apo enzyme, those in green are for the substrate-bound enzyme, and those in yellow are for both. Clearly, the ligated version has significantly more contacts. Among them, Q119 (in black) exhibits a shift that might be a marker for recrystallization. As mentioned in Fig. 7, it is also in a region where solution NMR shows a shift.

Fig. 9.
Activity assay of TIM in the direction of glyceraldehyde 3-phosphate to DHAP.

Table 1
Residues that have chemical shift perturbations between solid-state and solution NMR assignments

Residue	Thr4	Asn28	Thr29	Ser31	Asn35	Thr60	Gly128	Gly232
2° structure	Sheet 1	Loop after helix 1	Loop after helix 1	Loop after helix 1	Loop after helix 1	Sheet 3	Loop 5	Loop 8
$\gamma(\text{ppm})^a$	1.1 (N)	-0.5 (CO)	-0.4 (C^β)	-0.5 (C^β)	-0.5 (CO)	0.6 (C^β); -1.6 (N)	-1.2 (N)	-0.4 (C^α)

^aThe chemical shift difference is calculated relative to the mean in the Gaussian curves.