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Solid-State Nuclear Magnetic Resonance Studies Delineate the Role of the Protein in Activation of Both Aromatic Rings of Thiamin

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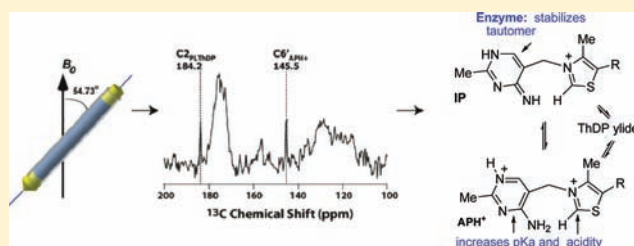
Supporting Information

ABSTRACT: Knowledge of the state of ionization and tautomerization of heteroaromatic cofactors when enzyme-bound is essential for formulating a detailed stepwise mechanism via proton transfers, the most commonly observed contribution to enzyme catalysis. In the bifunctional coenzyme, thiamin diphosphate (ThDP), both aromatic rings participate in catalysis, the thiazolium ring as an electrophilic covalent catalyst and the 4′-aminopyrimidine as acid−base catalyst involving its 1′,4′-iminopyrimidine tautomeric form. Two of four ionization and tautomeric states of ThDP are well characterized via circular dichroism spectral signatures on several ThDP superfamily members. Yet, the method is incapable of providing information about specific proton locations, which in principle may be accessible via NMR studies. To determine the precise ionization/tautomerization states of ThDP during various stages of the catalytic cycle, we report the first application of solid-state NMR spectroscopy to ThDP enzymes, whose large mass (160,000−250,000 Da) precludes solution NMR approaches. Three de novo synthesized analogues, [C2,C6′-$^{13}C_2$]ThDP, [C2-^{13}C]ThDP, and [N4′-^{15}N]ThDP used with three enzymes revealed that (a) binding to the enzymes activates both the 4′-aminopyrimidine (via pK_a elevation) and the thiazolium rings (pK_a suppression); (b) detection of a pre-decarboxylation intermediate analogue using [C2,C6′-$^{13}C_2$]ThDP, enables both confirmation of covalent bond formation and response in 4′-aminopyrimidine ring's tautomeric state to intermediate formation, supporting the mechanism we postulate; and (c) the chemical shift of bound [N4′-^{15}N]ThDP provides plausible models for the participation of the 1′,4′-iminopyrimidine tautomer in the mechanism. Unprecedented detail is achieved about proton positions on this bifunctional coenzyme on large enzymes in their active states.



INTRODUCTION

Our current understanding of thiamin diphosphate (ThDP, the vitamin B1 coenzyme) catalysis in biology suggests a mechanism, which incorporates concepts of electrophilic covalent catalysis and acid−base catalysis.[1] In fact, the ThDP superfamily of enzymes is so far virtually unique in utilizing such bifunctionality in a coenzyme: acid−base catalysis via the 4′-aminopyrimidine ring, and umpolung catalysis by the thiazolium ring, which together catalyze both 2-oxoacid decarboxylation and C−C carboligation reactions.[2−6] Key to the proposed catalytic mechanisms, are some critical protonation/deprotonation steps, which lead to activation of these rings (Scheme 1A). Defining the position of labile protons on aromatic cofactors such as ThDP at each step is essential to a fundamental understanding of acid−base catalysis on enzymes, and the current state of the art in X-ray crystallography precludes such understanding, as do solution state NMR studies in the case of such large proteins (M_r > 100,000). We here present the first solid-state NMR investigation to probe mechanistic issues in three members of the ThDP superfamily of enzymes, varying in molecular mass from 200 to 250 kDa.

Biological solid-state NMR spectroscopy has developed rapidly to become one of the essential biophysics and structural biology techniques.[7,8] The wealth of structural and dynamics information that can be derived from various NMR observables with unprecedented atomic-level detail, in conjunction with *independence of resonance line widths on the molecular size*, make solid-state NMR an especially powerful and attractive tool for investigations of large proteins and protein assemblies.[9−11] The potential of solid-state NMR in enzymology has remained relatively unexplored, with few applications to studies of enzyme mechanisms.[12−19]

As with any spectroscopic method, model systems are required for each state to be studied: three labeled analogues ([C2, C6′-$^{13}C_2$]ThDP, [C2-^{13}C]ThDP, and [N4′-^{15}N]ThDP) were synthesized de novo to provide reference values for different ionization and tautomerization states of ThDP on enzymes. Our working hypothesis is that: (1) protonation at the N1′ position of the 4′-aminopyrimidine ring (AP form) to the 4′-aminopyrimidinium (APH$^+$) must precede tautomerization to the 1′,4′-iminopyrimidine (IP form); (2) the IP form then assists ionization at the thiazolium C2-H position producing the thiazolium

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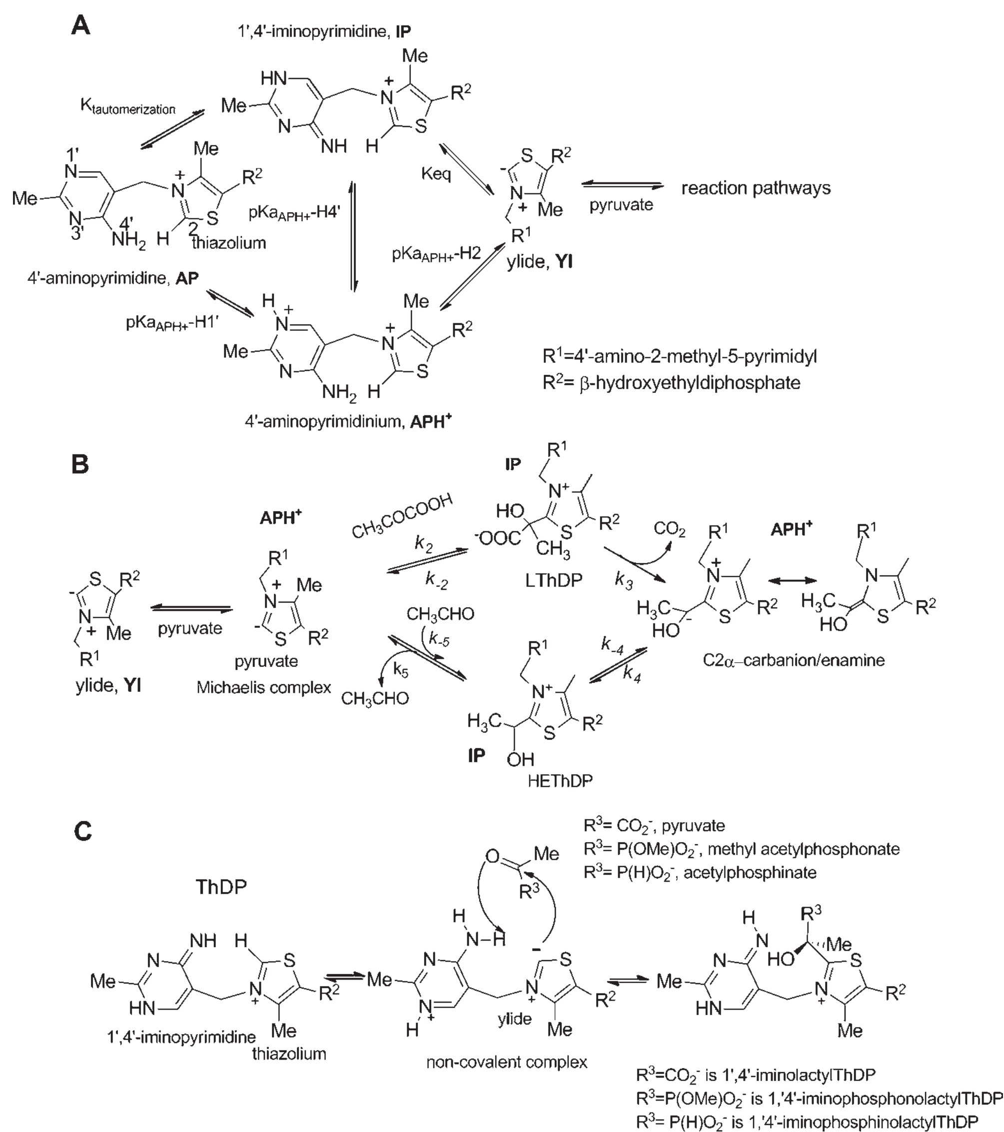
Scheme 1. (A) States of Tautomerization and Ionization of ThDP; (B) Mechanism of Pyuvate Decarboxylase; (C) Formation of Pre-decarboxylation Covalent Intermediate with ThDP from Pyruvate, Methyl Acetylphosphonate, and Acetylphosphinate



ylide (Yl), a central intermediate in all ThDP-dependent catalysis. The Rutgers group has shown on 10 enzymes to date that circular dichroism (CD) spectroscopy offers signatures [well characterized and supported with corresponding chemical models[20−22]] for identification of the AP and IP ThDP tautomeric forms (Scheme 1A). CD is useful, since all species of ThDP are chiral when enzyme bound. However, there is no conclusive CD evidence so far for the enzyme-bound APH^+ or Yl forms. As depicted in Scheme 1A, the APH^+ ionization state should predominate at pH values below its pK_a, while the AP, IP, and Yl forms should coexist predominantly at pH values above the pK_a; these three forms are all derived from APH^+ by proton loss at N1′, N4′, or C2, respectively. The Yl forms a covalent bond with the substrate, while the APH^+ form, along with the IP form, are necessary and sufficient to completely describe all ThDP pathways (see Scheme 1B for an example of a 2-oxoacid decarboxylase). Surprisingly, the presence of the canonical AP form is neither sufficient nor necessary to describe these pathways.

Elsewhere, we reported assignment of the ^{13}C and ^{15}N isotropic chemical shifts as well as the complete chemical shift anisotropy tensors of key atoms (C2, C6′, and N4′) of ThDP (Supporting Information, Table S1) in different states of ionization of the 4′-aminopyrimidine ring.[23] With these assignments in hand, here we could interpret ^{13}C and ^{15}N NMR results on ThDP bound to: (1) recombinant yeast pyruvate decarboxylase from *Saccharomyces cerevisiae* overexpressed in *E. coli* (YPDC, EC 4.1.1.1); (2) first enzymatic component of the *E. coli* pyruvate dehydrogenase complex (E1p, EC 1.2.4.1), and (3) the first enzymatic component of the *E. coli* 2-oxoglutarate dehydrogenase complex (E1o, EC 1.2.4.2). The results provide the following novel information for ThDP-catalyzed reactions: (1) A change in chemical shift of the thiazolium C2 atom upon binding to the three enzymes, indicating activation of this position by the protein, not observed in solution NMR studies;[24] (2) The stabilization of the APH^+ form on all three enzymes prior to substrate addition, signifying that the enzymes activate the 4′-aminopyrimidine ring by pK_a elevation at the N1′ position

assuring the presence of all tautomeric/ionization forms near the pH optimum. (3) Chemical shift evidence for formation of a tetrahedral pre-decarboxylation intermediate with substrate analogues on E1p and YPDC. The [C2,C6′-$^{13}C_2$]ThDP enables for the first time monitoring both covalent adduct formation at the C2 thiazolium atom and its consequence on the state of ionization/tautomerization of the 4′-aminopyrimidine ring. (4) Chemical shift of the ^{15}N resonances pertinent to the bound N4′ atom in a pre-decarboxylation intermediate; the observed chemical shift clearly indicates a significant contribution from the IP form. In this study, the enzyme complex is precipitated as it would be under crystallization conditions, but at the pH optimum of the enzyme, the intermediates are observed while enzyme bound.

MATERIALS AND METHODS

Materials. MES, Tris·HCl, ThDP, and DTT were from USB. ATP was from Sigma-Aldrich (St. Louis, MO). Optimized for crystallization, polyethylene glycols PEG 8000 (flakes), PEG 4000 (flakes), PEG monomethyl ether 2000 (50% solution), and 2-propanol were from Hampton Research (Aliso Viejo, CA). PEG 2000 monomethyl ether 2000 (flakes) crystallization grade was from Fluka Analytical (Buchs SG, Switzerland).

Syntheses. [C2,C6′-$^{13}C_2$]Thiamin, [N4′-^{15}N]thiamin, methyl acetylphosphonate sodium salt (MAP), acetylphosphinate sodium salt (AcPhi), and pyruvamide were synthesized as reported elsewhere.[21,23] [C2-^{13}C]Thiamin was a kind gift from Dr. John Schloss (currently at University of New England, Biddeford and Portland, ME). All thiamin analogues were converted to ThDP as described in detail in the Supporting Information (SI).

Protein Purification. YPDC, E1p, and E1o were purified as described elsewhere[25,26] and the procedures employed to convert these enzymes to their respective apoenzymes and their subsequent reconstitution with labeled ThDP are described in detail in SI.

NMR Samples and Spectroscopy. The enzymes free of unbound labeled cofactors (E1o and YPDC; E1p sample solutions contained three active-site equivalents of ThDP at this stage) prepared in buffers to attain the necessary pH were incubated with MAP, AcPhi, or pyruvamide. Next, the enzymes were precipitated with their respective crystallization buffers, and the precipitates were collected and loaded into zirconium SSNMR rotors.

All ^{13}C CPMAS (cross-polarization magic angle spinning) spectra were acquired at 9.4 T (400.17 MHz ^{1}H Larmor frequency) on a Tecmag Discovery spectrometer outfitted with a 3.2 mm wide bore Varian HXY T3 probe. The MAS frequency was 10.000 ± 0.010 kHz controlled by a Tecmag MAS controller. The temperature calibration was done using a $PbNO_3$ temperature sensor. For both ^{13}C and ^{15}N CPMAS experiments, the actual sample temperature includes a correction of +5 °C taking into account sample spinning. ^{13}C chemical shifts were referenced to the downfield peak of adamantane (38.56 ppm with respect to TMS). The ramped CP sequence was used with 1.0−1.5 ms contact time. The ^{1}H radio frequency field strength was 50−60 kHz, and the ^{13}C field was linearly ramped from 80 to 100% with the center of the ramp being $\omega_{rf}(^{13}C) = \omega_{rf}(^{1}H) \pm \omega_r$. The ^{1}H 90° pulse length was 2.5−3.65 μs. The ^{1}H decoupling was performed using TPPM[27] with ω_{rf} = 70−100 kHz. The number of transients and the recycle delay for each spectrum are given in the figure captions. All ^{13}C spectra were processed with 20 Hz exponential broadening.

All ^{15}N CPMAS spectra were acquired at 14.1 T (599.78 MHz ^{1}H Larmor frequency) on a Varian InfinityPlus spectrometer outfitted with a standard bore 3.2 mm HXY T3 probe. The MAS frequency was 10.000 ± 0.001 kHz controlled by a Varian MAS controller. ^{15}N chemical shifts were referenced to ammonium chloride (39.2 ppm with respect to liquid NH_3). The ramped CP sequence was used with 1.5−1.6 ms contact time. The ^{1}H radio frequency field strength was 50−60 kHz, and the ^{15}N field was linearly ramped from 80 to 100% with the center of the ramp being $\omega_{rf}(^{15}N) = \omega_{rf}(^{1}H) \pm \omega_r$. The ^{1}H 90° pulse length was 2.50−2.78 μs. The ^{1}H decoupling was performed using TPPM [27] with ω_{rf} = 90−100 kHz. The number of transients, the recycle delay, and the apodization parameters for each spectrum are given in the figure captions.

CD Spectroscopy. Near-UV (280−400 nm) CD spectra were acquired using an Applied Photophysics (Leatherhead, Surrey, UK) Chirascan instrument equipped with a quartz cuvette of 1 cm path length and the temperature at 25 °C. To study the pH dependence over the range (6.59−7.85) of the spectral bands, the pH of the solution was adjusted to the desired value with either 1 M acetic acid or 1 M Tris base measured by a sympHony pH electrode (VWR), and CD spectra were recorded after each adjustment. (Inset) pH dependence of the CD bands at 320 nm reflecting AP formation. The pK_a was determined using the equation: $\log(Y) = \log(Y_{max}) - \log(1 + 10^{(pK_a - X)})$ where Y and Y_{max} are CD and CD_{max}.

RESULTS

Enzymes Activate ThDP by Stabilization of the APH^+ State and Perturbation of Thiazolium C2-H Bond. When the ^{13}C CPMAS solid-state NMR (SSNMR) spectrum of YPDC reconstituted with [C2,C6′-$^{13}C_2$]ThDP was compared to the spectrum of YPDC with unlabeled ThDP, two new resonances stood out: one at 162.2 ppm, and another at 146.5 ppm, the latter reminiscent of the C6′ carbon in protonated form of thiamin (a and b of Figure 1). On the basis of this experiment, as well as experiments on E1o reconstituted with [C2,C6′-$^{13}C_2$]ThDP (Figure 2) and E1p reconstituted with [C2-^{13}C]ThDP (Figure 4a) discussed later, the resonances could be assigned to individual atoms of ThDP bound to YPDC: the one at 162.2 ppm to C2 and the one at 146.5 ppm to C6′ in the APH^+ form. In Table S1 (SI) and ref 23 are provided for comparison relevant model chemical shifts, recorded in the solid state on the AP and APH^+ forms of the corresponding thiamin compounds.

On the basis of these observations we draw the following conclusions: (a) ThDP is in its APH^+ form according to the chemical shift of the C6′ atom, and (b) the C2-H bond of the thiazolium ring is undissociated. No resonance was in evidence near 250 ppm, previously assigned to a YI model by solution NMR.[28] However, there is a significant chemical shift perturbation at C2 according to SSNMR, an approximately 6−9 ppm deshielding by YPDC (Figure 1b), a perturbation of the same magnitude being observed on E1p and E1o.

In a solution NMR study the C2 chemical shift of [C2-^{13}C]-ThDP bound to YPDC[24] was reported to be virtually unchanged compared to free ThDP, suggesting undissociated C2-H. Our attempts to observe solution NMR signals from [C2,C6′-$^{13}C_2$]-ThDP incorporated into YPDC were unsuccessful. Spectra of YPDC and apo-YPDC reconstituted with labeled ThDP appeared similar, with no discernible changes at either the ~146 ppm (C6′) or ~162 ppm (C2) region (see Figure S1in SI). We have no explanation for the difference between our results and those in ref 24, but do note that, for a protein with mass of >240 kDa, very broad lines are expected.

YPDC is known to be subject to homotropic activation by pyruvate binding at a distinct regulatory site Cys221.[29,30] Pyruvamide, a non-decarboxylating analogue, is a known substrate activator surrogate.[31] NMR-based H/D exchange experiments at

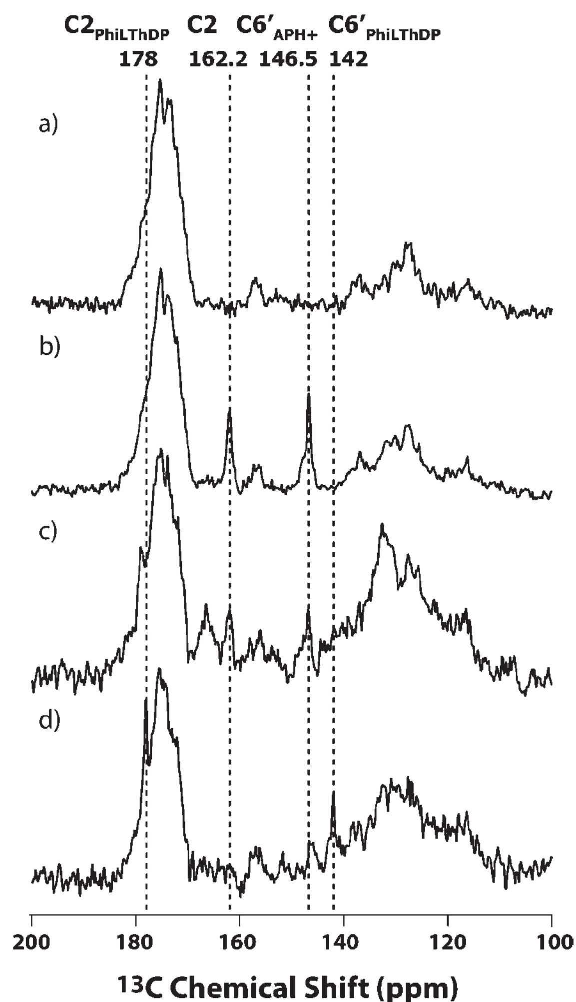


Figure 1. ^{13}C CPMAS SSNMR spectra of YPDC reconstituted with [C2,C6′-$^{13}C_2$]ThDP (a), YPDC with unlabeled ThDP (control spectrum) only (b), [C2,C6′-$^{13}C_2$]ThDP (c), [C2,C6′-$^{13}C_2$]ThDP and pyruvamide (100 mM). The broad resonance at ~166 ppm is from excess pyruvamide, (d) [C2,C6′-$^{13}C_2$]ThDP, and acetyl phosphinate (60 mM). All the spectra were acquired at 5 °C with 16,384 transients and 5 s recycle delay.

the thiazolium C2H position of ThDP (for estimating the rate of C2-H dissociation) signaled a 600-fold rate acceleration by pyruvamide.[24] In SSNMR experiments, addition of pyruvamide (200 mM, Figure 1c) to the apo-YPDC reconstituted with [C2, C6′-$^{13}C_2$]ThDP induced no changes in the isotropic chemical shifts of the C2 and C6′ atoms of ThDP; these were again found at 162.2 ppm and 146.5 ppm, respectively.

APH^+ State Is Stabilized Prior to Substrate Addition by pK_a Elevation at the N1′ Position. The solid-state isotropic chemical shift of the C6′ atom provided direct evidence for the presence of the APH^+ form on all three enzymes tested, we next determined the mechanism of stabilization of this ionization state. In CD experiments only the AP and IP forms could be detected, and presence of the APH^+ form could only be inferred from the pH titration of the AP form;[20] Such a CD-detected titration enabled estimation of a pK_a of 7.2 for the APH^+ form on E1o (Figure 2, bottom). The SSNMR findings with C6′ are complementary to the CD results, consequently increasing the pH should decrease the relative concentration of the APH^+ form,

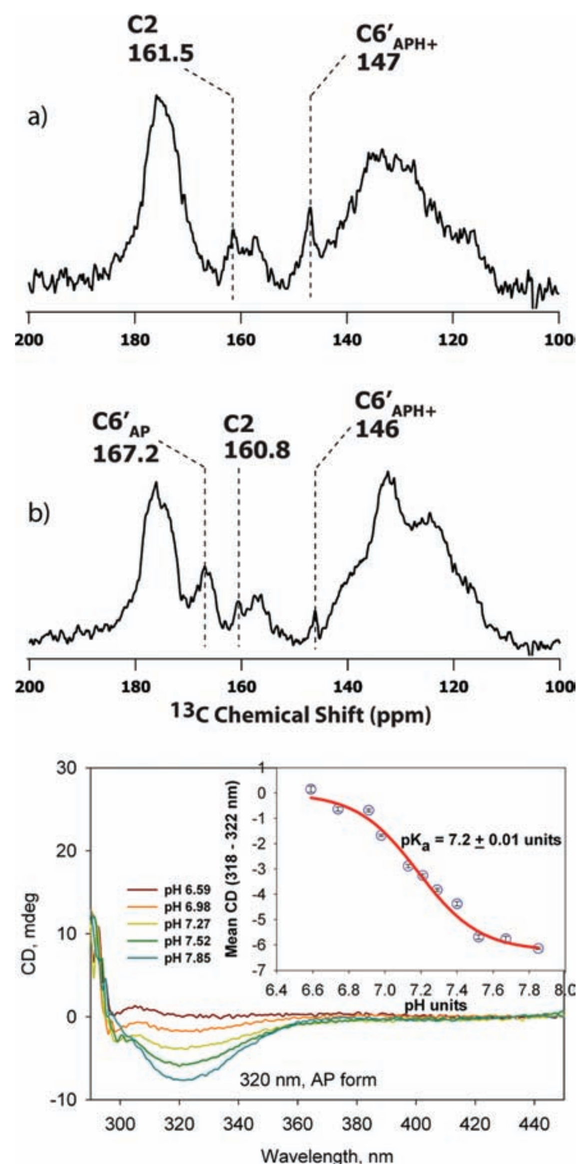


Figure 2. ^{13}C CPMAS SSNMR spectra of E1o reconstituted with [C2, C6′-^{13}C2]ThDP. (a) Spectrum acquired at pH 7.0 at 8 °C with 32,768 transients. (b) Spectrum acquired at pH 8.0 at 10 °C with 28,900 transients. Recycle delay was 5 s for acquiring both spectra. (Bottom) Representative near-UV CD spectra of E1o at varying pH values. E1o (5 mg/mL) in 2.4 mL of a triple buffer (15 mM MES, 15 mM KH_2PO_4, and 15 mM Tris) containing additional 0.5 mM ThDP, and 2.5 mM Mg^{2+}.

and only the C6′ resonance should be affected. Indeed, ^{13}C SSNMR spectra of E1o reconstituted with [C2,C6′-$^{13}C_2$]ThDP at pH 7.0 and pH 8.0 (Figure 2) revealed that the resonance at 146−147 ppm pertaining to the C6′ carbon diminishes in intensity significantly with increasing pH (relative to protein backbone), and a broad new resonance is seen at 167.2 ppm which is assigned to C6′ of enzyme-bound ThDP in the AP form. This experiment provides further evidence that the pK_a of APH^+ is elevated within the active sites of ThDP enzymes, and for E1o it is 7.2 (from the CD experiment), compared to 4.85 in water for the free thiamin. While there is evidence for a difference of 0.9 pK_a units for the active center histidine of α-lytic protease measured in solution and solid state, the same group determined by solution NMR that 0.6 units of that difference was due to the use of

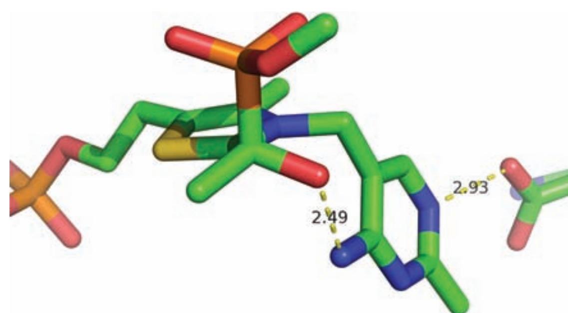


Figure 3. C2-α-PhosphonolactylThDP (PLThDP), a tetrahederal intermediate analogue bound to the active site of E1p. Illustration created using the graphics program PyMOL from the PDB file PDB ID: 2G25. Interatomic distances between N4′ and C2α-O atoms (2.49 Å) and N1′ and terminal COO^- of Glu571 (2.93 Å) were measured using the distance measurement tool in the software.

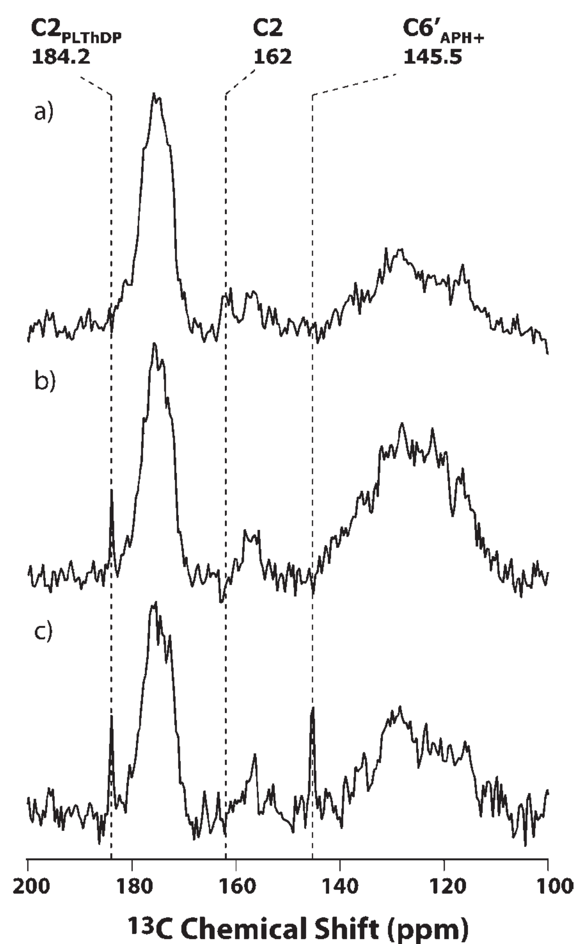


Figure 4. ^{13}C CPMAS SSNMR spectrum of E1p reconstituted with [C2,C6′-$^{13}C_2$]ThDP or [C2-^{13}C]ThDP. E1p reconstituted with (a) [C2-^{13}C]ThDP, (b) [C2-^{13}C]ThDP and additional 10 mM methyl acetylphosphonate, (c) [C2,C6′-$^{13}C_2$]ThDP and additional 10 mM methyl acetylphosphonate. The spectra were acquired at 15 °C with 16,384 transients; the recycle delay was 10 s (spectrum a) and 5 s (spectra b and c).

0.6 M Li_2SO_4.[14] Our precipitation buffers do not contain such large concentration of salt. Also, our analysis depends on pH-dependent trends in relative resonance magnitudes, and we are not using SSNMR to quantify pK_a, for which CD is preferred and is simpler (Figure 2, bottom).

Formation of a Stable Pre-decarboxylation Intermediate Analogue at the C2 Atom and Its Consequence at the 4′-Aminopyrimidine Ring Can Be Observed Simultaneously. At Rutgers, 2-oxophosphonates and 2-oxophosphinates have been used as excellent 2-oxoacid analogues that form stable pre-decarboxylation intermediate analogues.[32] In the first instance, methyl acetylphosphonate (MAP, single negative charge at the phosphonate monoester) was used on ThDP enzymes, and with E1p from *E. coli* the covalent pre-decarboxylation intermediate adduct C2α-phosphonolactylthiamin diphosphate (PLThDP) (Scheme 1C) could be identified by X-ray crystallography.[25] Interatomic distances measured between C2α-O and N4′ atoms in this complex (Figure 3), and on similar complexes are unusually short (see Table S2 in SI) when compared to the normal range for O···N H-bonding distances (2.7−3.0 Å). Also, acetylphosphinate (AcPhi), an even better steric approximation to pyruvate, was shown on several ThDP enzymes to form such complexes and also to behave as potent inhibitor.[33] Both pyruvate analogues gave strong CD evidence that, in their covalent adducts with ThDP on enzymes, the IP tautomeric form predominates. We report further evidence for the environment around individual atoms in these covalent intermediate analogues using SSNMR spectroscopy.

Addition of MAP to E1p Resulted in the Spectra in b and c of Figure 4. Two experiments were carried out in the presence of MAP to facilitate the assignments: the middle spectrum (Figure 4b) is with the singly labeled [C2-^{13}C]ThDP, the bottom spectrum is with the doubly labeled [C2,C6′-$^{13}C_2$]ThDP (Figure 4c). The spectra provide unambiguous assignment of C2 and C6′, 184.2 ppm for the former and 145.5 ppm for the latter in the presence of MAP. The C2 resonance at 184.2 ppm displays a 22 ppm deshielding on formation of the tetrahedral PLThDP, appropriate for replacement of H by C.[34] Acid quench of a mixture of E1p reconstituted with [C2,C6′-$^{13}C_2$]ThDP and MAP followed by solution 1D-^{1}H gCHSQC NMR analysis of the supernatant revealed the presence of PLThDP at ~80% occupancy in the active site, confirming formation of PLThDP (SI Figure S2).

Addition of AcPhi to YPDC Resulted in the Spectrum in Figure 1d. Three prominent changes were observed. (1) The C2 resonance at 162 ppm disappears. (2) Two new resonances at 142 ppm and 178 ppm appear. (3) The resonance at 146.5 ppm for the C6′ in the APH$^+$ form of ThDP is significantly diminished. Similar to the E1p−MAP experiment, the disappearance of the 162 ppm resonance, and the appearance of a new one at 178 ppm could be attributed to the C2 atom in the enzyme-bound C2-α-phosphinolactylThDP intermediate (PhiLThDP, C2-α-phosphinolactylthiamin diphosphate the adduct of acetyl phosphinate and ThDP). The most novel finding is the strong resonance at 142 ppm, coexisting with the weaker yet clear resonance at 146.5 ppm. First, the resonance at 146.5 ppm (C6′) corresponds to a fraction of enzyme-bound ThDP not forming a C2 adduct, but seen as a Michaelis complex in CD experiments on YPDC,[21] while the resonance at 142 ppm pertains to the C6′ resonance of the 4′-aminopyrimidine ring of PhiLThDP. The chemical shift for the C6′ resonance in the IP form is 136 ppm according to model studies by solution NMR, while the observed chemical shift is a value in-between those for the IP (136 ppm) and the APH$^+$ (146.5 ppm) forms. The C2 chemical shifts enabled direct detection of the enzyme-bound tetrahedral intermediate of ThDP (C2α-lactylthiamin diphosphate-like

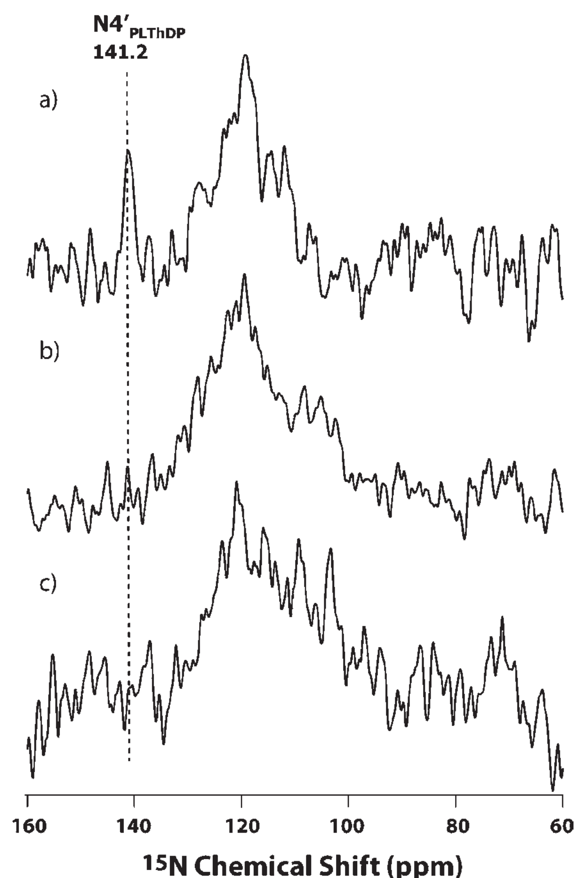


Figure 5. ^{15}N CPMAS SSNMR spectra of E1p reconstituted with [N4′-^{15}N]ThDP at pH 7.0. E1p reconstituted with: (a) 3 equiv [N4′-^{15}N]-ThDP and additional 10 mM methyl acetylphosphonate at −25 °C. (b) 3 equiv [N4′-^{15}N]ThDP at −25 °C. (c) 1 equiv [N4′-^{15}N]ThDP at −23 °C. The spectra are a sum of 30,720 transients (spectrum a), 16,384 transients (spectrum b), and 14,336 transients (spectrum c); the recycle delay was 5 s. All spectra were processed with 50 Hz exponential broadening.

[LThDP]) in B and C of Scheme 1, while the C6′ resonance illuminated the consequence of C2-adduct formation on the 4′-aminopyrimidine ring.

Role of the N4′ Atom during the Catalytic Cycle. *Observation of the N4′ Atom on E1p.* We hypothesized that observation of the ^{15}N resonance from specifically ^{15}N-labeled [N4′-^{15}N]-ThDP would provide the most direct evidence for the state of tautomerization/ionization of the 4′-aminopyrimidine ring. This experiment is even more challenging than the ^{13}C experiments above because of the lower S/N ratios even at 14.1 T (^{1}H Larmor frequency of 600 MHz). First, E1p was reconstituted with [N4′-^{15}N]ThDP. The ^{15}N resonance of [N4′-^{15}N]ThDP in the APH^{+} ionization state in the free ThDP appears at 116.5 ppm (Table S1 in SI and ref 22), which falls within the envelope of the enzyme backbone amide resonances. While the enzyme-bound ThDP is predicted to be partially in the APH^{+} form at pH 7.0 according to ^{13}C experiments, no clear evidence for a ^{15}N resonance pertaining to the enzyme-bound APH^{+} form could be obtained. In the presence of MAP, we observe clear indication of a new signal around 141.2 ppm at pH 7.0 (Figure 5), a chemical shift in between the 212 ppm for the IP form and 116.5 ppm for the APH^{+} form (it is 91.2 ppm for the AP form), from models in the absence of enzyme (Table S1 in SI).

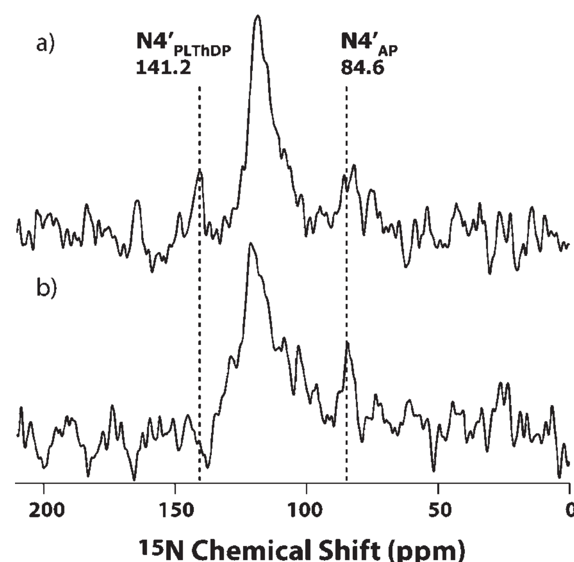


Figure 6. ^{15}N CPMAS SSNMR spectrum of E1p reconstituted with [N4′-^{15}N]ThDP at pH 8.0. E1p reconstituted with **a)** 3 equiv [N4′-^{15}N]ThDP and additional 10 mM methyl acetylphosphonate. **b)** Reconstituted with 3 equiv [N4′-^{15}N]ThDP. The spectra were acquired at −25 °C with 32,768 scans and 5 s recycle delay. Both spectra were processed with 100 Hz exponential broadening.

The pH dependence of CD bands on the ThDP enzyme family,[20] and the pH-dependent ^{13}C SSNMR experiments on E1o predict a shift from APH^{+} to the AP or IP forms (two forms whose molar ratio should be pH-independent, hence should display the same behavior with pH) for the enzyme-bound ThDP at higher pH values. Raising the pH from 7.0 to 8.0 gives clear indication for the existence of the AP form (84.6 ppm) of the enzyme-bound ThDP in E1p (see Figure 6). In the presence of MAP at pH 8.0, we observe two resonances, one at 84.6 ppm (AP form) and one at 141.2 ppm. Simultaneous observation of these two resonances suggests that the AP form is in slow exchange (essentially does not exchange) with the other two forms, while the IP and APH^{+} forms are likely in fast protolytic equilibrium with each other. Similar chemical shift averaging due to fast exchange is reported on pyridoxal model systems, while averaging between two-site and three-site equilibria are seen by SSNMR studies on the pyridoxal-5′-phosphate dependent tryptophan synthase when the active-site is occupied by a quinonoid intermediate.[18,35]

Observation of the N4′ Atom on YPDC. The spectra of YPDC reconstituted with [N4′-^{15}N]ThDP recorded at pH 6.0 (Figure 7) gave no evidence of a bound ^{15}N resonance pertaining to the APH^{+} form as suggested by ^{13}C NMR, either in the absence or presence of pyruvamide (Figures 7b and 7c). On addition of acetylphosphinate, there is evidence of a signal centered at 176 ppm, arising from the N4′ nucleus (Figure 7a), similar to the observation with E1p, but with greater deshielding.

DISCUSSION

We present the first observation by SSNMR of the states of ionization and tautomerization of ThDP bound to three enzymes by monitoring the key carbon and nitrogen atoms specifically introduced into the coenzyme. The interpretations were made possible by synthesis of chemical models for the various ionization/tautomerization states.[22,23] Although we do have appropriate model chemical shifts for the three (APH^{+}, IP, and AP) environments,

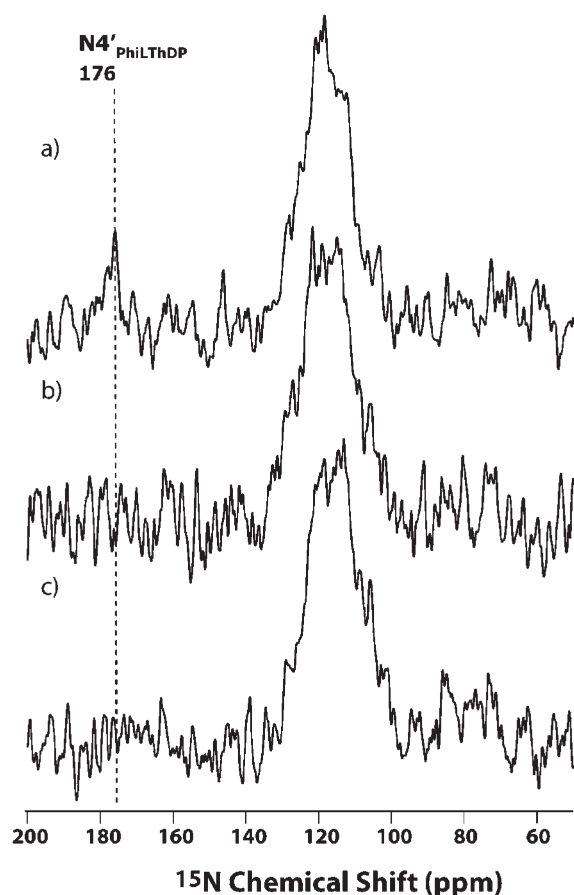
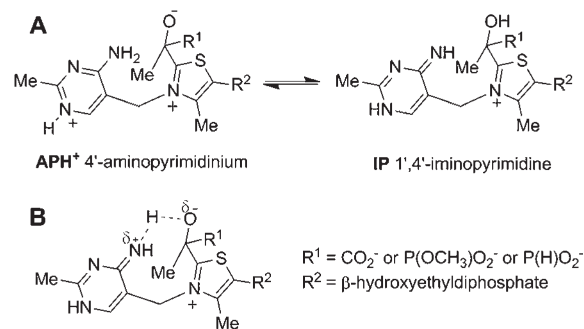


Figure 7. ^{15}N CPMAS SSNMR spectrum of YPDC reconstituted with [N4′-^{15}N]ThDP. YPDC reconstituted with [N4′-^{15}N]ThDP: (a) in the presence of 60 mM acetyl phosphinate at −25 °C; (b)in the presence of 300 mM pyruvamide −25 °C; (c) [N4′-^{15}N]ThDP −25 °C. To obtain (a) 57,344 transients with 4 s recycle delay, (b) 23,552 transients with 5 s recycle delay, and (c) 27,648 transients with 5 s recycle delay, 50 Hz exponential broadening was used.

the impact of the enzyme environment on the chemical shift of ^{15}N resonances (especially for strongly hydrogen-bonded systems and systems probably undergoing chemical exchange) is difficult to predict and equally difficult to model. While models for APH$^+$ and AP are based on SSNMR measurements on thiamin itself, those for the IP form were generated in solution for 4′-aminopyrimidine derivatives, lacking both the thiazolium ring and substituent at the C2 carbon.

ThDP bound to many members of the ThDP superfamily was found to be in the activated state as judged by the enhanced C2-H/D exchange rates compared to the free coenzyme.[24] Moreover, this step is not rate determining in most ThDP-dependent enzymes. Our observation of the APH$^+$ state in all three enzymes in the absence of substrate near the optimal pH of the enzymes provides direct evidence for the initial step in the proposed mechanism of ThDP activation. On the E1o and E1p enzymes, the C6′ resonance and N4′ resonances, respectively, respond to pH change. This is consistent with the idea that the pK_a for the APH$^+$ is significantly elevated on the enzymes (compared to 4.85 in water[36]) due to the following: (i) an electrostatic interaction with the highly conserved glutamate residue within hydrogen-bonding distance of the N1′ atom and (ii) the active sites favoring a positive charge at the N1′ position. This pK_a elevation is also a requirement for catalytic efficiency since the other forms (IP and Yl) must be derived from APH$^+$ chemically (Scheme 1A).

Scheme 2. Plausible Explanations of Observed ^{15}N Chemical Shift; (A) Fast Exchange of Proton between Two Environments, 4′-NH_2 and C2α-OH; (B) Proton Located between N4′ and C2α-O



The chemical shift of 162 ppm for the C2 atom of the enzyme-bound ThDP, as compared to 153.4−156.5 ppm for free ThDP, suggests that the catalytic center environments induce modestly increased acidity (according to the deshielding) on all three enzymes. The deshielding may also be due to chemical exchange among species in Scheme IA, including Yl, and cannot be ruled out by our data (cf. enhanced C2-H/D exchange rates). The changes in chemical shift at C2 on addition of the substrate analogues provide strong independent and unambiguous evidence for the formation of a tetrahedral intermediate on two enzymes, previously deduced from X-ray structures. Other covalent intermediates such as the enamine could also be studied by this method under appropriate conditions, as NMR chemical shift is a very sensitive measure of changes in bond order.

A challenge ahead is further elucidation of the behavior of the 4′-^{15}N resonance, observed clearly only when there is a tetrahedral pre-decarboxylation complex at the C2 atom of ThDP. We propose the following two plausible explanations consistent with the observed chemical shift of the ^{15}N resonance: (a) There is a fast pH-dependent chemical exchange (Scheme 2A) between IP (212 ppm in models) and APH$^+$ (115 ppm in models) forms. Assuming that the observed signal represents a weighted average of the two forms, one can estimate a pK_a of 5.73 for the APH$^+$ form on YPDC. This is similar to the pH optimum of the enzyme,[26] consistent with our earlier finding that the pK_a of the APH$^+$ form on all ThDP enzymes studied to date is in the middle of the optimum pH range of activity, always higher than in water. (b) Alternatively, the proton influencing the ^{15}N chemical shift is located in between (is shared by) the N4′ nitrogen atom and the C2α-O oxygen atom, and the particular value observed with different enzymes depends on the location of this proton; the closer it is to oxygen (Scheme 2B), the more the observed chemical shift resembles the chemical shift of the 1′,4′-iminopyrimidine tautomeric form generated in models. Other residues hydrogen bonding to the N4′ would also affect its chemical shift, which could vary among enzymes as seen in the cases of E1p and YPDC. The short hydrogen bond observed in the PLThDP−E1p complex (2.49 Å) certainly would not preclude such a ‘bridging’ proton (Figure 3). Such an intermediate proton position would also be mechanistically useful, as this proton has to constantly shuttle during the reaction. As of this writing, the second option is marginally preferred, in view of the pH-independent ^{15}N chemical

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shift on (E1p+MAP), and the observed 142 ppm C6′ chemical shift on (YPDC+AcPhi). Studies at higher fields affording better sensitivity and temperature-dependent measurements covering broad temperature regimes will be required to further test our alternative explanations.

CONCLUSION

This study demonstrates that the states of ionization and tautomerization can indeed be ascertained on large enzymes (E1p and E1o of ~200 kDa, and YPDC of ~250 kDa) by a combination of MAS NMR and CD methods. Our observations suggest that enzyme-bound ThDP is activated by protonation of the 4′-aminopyrimidine ring and perturbation of the C2-H bond of the thiazolium ring. The activation of the 4′-aminopyrimidine ring is achieved by pK_a elevation at the N1′ position. In addition to its participation in ylide formation, the 4′-aminopyrimidine ring also participates in the interconversion of ThDP-bound covalent intermediates via its 1′,4′-iminopyrimidine tautomeric form. Finally, we suggest the possibility of participation in other catalytic processes of such rare tautomers of heteroaromatic rings/cofactors, including the nucleic bases in ribozymes, and perhaps in the ribosome.

ASSOCIATED CONTENT

Supporting Information. Synthesis and ^{13}C NMR chemical shifts of model for 1,4-iminopyrimidine; enzymatic synthesis of labeled thiamin diphosphate from thiamin; protein expression and purification methods; sample preparation for SSNMR studies; two solution NMR spectra of intermediates; table of reference chemical shifts; table of distances between N4′ and C2α-O atoms on stable pre-decarboxylation intermediate analogues reported in X-ray structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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