JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Protonation States of the Tryptophan Synthase Internal Aldimine Active Site from Solid-State NMR Spectroscopy: Direct Observation of the Protonated Schiff Base Linkage to Pyridoxal-5′-Phosphate

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Supporting Information

ABSTRACT: The acid−base chemistry that drives catalysis in pyridoxal-5′-phosphate (PLP)-dependent enzymes has been the subject of intense interest and investigation since the initial identification of PLP's role as a coenzyme in this extensive class of enzymes. It was first proposed over 50 years ago that the initial step in the catalytic cycle is facilitated by a protonated Schiff base form of the holoenzyme in which the linking lysine ε-imine nitrogen, which covalently binds the coenzyme, is protonated. Here we provide the first ^{15}N NMR chemical shift measurements of such a Schiff base linkage in the resting holoenzyme form, the internal aldimine state of tryptophan synthase. Double-resonance experiments confirm the assignment of the Schiff base nitrogen, and additional ^{13}C, ^{15}N, and ^{31}P chemical shift measurements of sites on the PLP coenzyme allow a detailed model of coenzyme protonation states to be established.

Pyridoxal-5′-Phosphate (PLP), the bioactive form of vitamin B_6, acts as a coenzyme in multiple amino acid transformations, including $\alpha/\beta/\gamma$ elimination/replacement, racemization, transamination, and decarboxylation.[1,2] At the start of the catalytic cycle, the cofactor is covalently attached to the enzyme through an imine bond to the ε-nitrogen of a lysine side chain, giving a secondary aldimine, or Schiff base, species called the "internal" aldimine (Figure 1). It has been proposed that a positively charged protonated Schiff base (PSB) tautomer activates catalysis at this point by forming a significantly more reactive target for nucleophilic attack than the neutral imine,[4−6] while the protonation states of other sites on the coenzyme—the phenolic oxygen and PLP ring nitrogen in particular—are thought to be critical in establishing the specificity of the reaction pathway.[2,7−9] The experimental determination of protonation states within PLP active sites is typically indirect; the two most common methods of characterization, X-ray crystallography and optical spectroscopy, cannot specifically identify proton locations or report unambiguously on the local chemical environment of individual atoms. NMR spectroscopy can provide atomic-resolution characterization, but to date only a handful of NMR studies of PLP-dependent enzyme active sites have been reported.[10−14] Here we detail ^{15}N NMR chemical shift measurements of the Schiff base linkage in the resting holoenzyme state of a PLP-dependent enzyme, the tryptophan synthase internal aldimine complex; these provide the first direct atomic-resolution observation of the protonated Schiff base tautomer by NMR spectroscopy. Additional ^{13}C, ^{15}N, and ^{31}P chemical shift measurements on the coenzyme allow a chemically detailed model—including all of the coenzyme protonation states—to be established.

The tryptophan synthase (TS) $\alpha_2\beta_2$ bienzyme complex relies on PLP to bring together indole and L-serine to form L-tryptophan.[15−18] Figure 1 shows a schematic of the β-subunit active site for the internal aldimine state, E(Ain), with PLP covalently bound to βLys87. In TS (as with all PLP-dependent enzymes), the first step in catalysis is a transimination reaction in which an amino acid substrate makes a nucleophilic attack at C4′

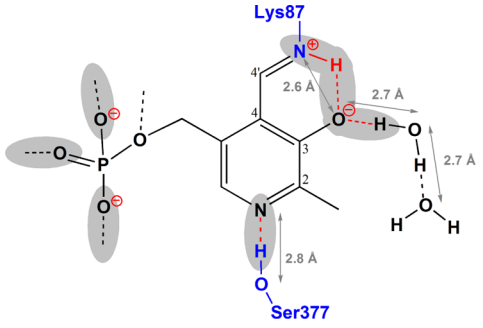

Figure 1. Schematic of PLP covalently bound to βLys87 in the β-subunit active site of tryptophan synthase. Distances and water molecules are from the X-ray crystal structure of the *S. typhimurium* TS internal aldimine state (PDB code 4HT3). Possible sites of protonation/ionization on the coenzyme and linking imine nitrogen are indicated in gray, along with hydrogen bonds to active-site residues/water. The donor and acceptor atoms of these hydrogen bonds are able to switch roles (potentially requiring another proton) to stabilize multiple, alternate protonation states, including the deprotonated Schiff base nitrogen/protonated phenolic oxygen pairing and/or protonation at the pyridine nitrogen (N1). The experimentally determined protonation states from solid-state NMR spectroscopy are highlighted in red. Protein residue fragments are shown in blue and PLP and water in black.

Received: June 22, 2014
Published: August 22, 2014

   dx.doi.org/10.1021/ja506267d | J. Am. Chem. Soc. 2014, 136, 12824−12827

of PLP; this step exchanges the PLP Schiff base linkage to the protein with one to the substrate. The PSB hypothesis posits that the imine nitrogen should be protonated for reactivity toward nucleophiles at the Schiff base carbon, a hypothesis first proposed to explain the remarkable acceleration of enzyme-catalyzed reactions compared with PLP-catalyzed reactions in solution.[4]

UV/vis optical spectroscopy of TS supports the PSB hypothesis. Studies of enzyme internal aldimine states and model compounds with Schiff base linkages to peptides and amino acids in polar, aprotic solvents indicate a conjugation best explained by a protonated Schiff base nitrogen.[5,6,19] The internal aldimine complexes of most PLP enzymes give absorption maxima in the 420 to 430 nm range, and the 412 nm λ_{max} exhibited by the TS E(Ain), both in solution and in single crystals, correlates well with the postulate of a Schiff base structure conjugated with the PLP π system.[20] The UV/vis spectrum of TS is independent of pH over the range 5.8−10.4,[20−22] and consequently, there is a single ionic form of the E(Ain) β-site and no change in the distribution of tautomeric structures in this pH range.[23] Deprotonation of PLP enzyme Schiff bases causes shifts to shorter wavelength (~360 nm),[24] while deprotonation of the internal aldimine PLP ring nitrogen has only minor effects on the UV/vis spectrum.[25]

The X-ray crystal structures of TS internal aldimine complexes show the Schiff base nitrogen in close proximity to the 3′ oxygen of the PLP ring.[26−31] Key distances from the 1.30 Å resolution X-ray crystal structure of the *Salmonella typhimurium* TS E(Ain) (reported recently by several of us;[31] PDB accession code 4HT3) are shown in Figure 1. The 2.6 Å Schiff base nitrogen to phenolic oxygen distance is fully consistent with the presence of a proton in an N−H−O hydrogen bond, but the assignment of donor and acceptor in this bonding system cannot be established directly from the crystal structure. The phenolic oxygen is also involved in a hydrogen-bonding network with two crystallographically observed water molecules, as indicated in the figure.

While X-ray crystallography and UV/vis spectroscopy suggest a protonated Schiff base form, atomic-resolution probes, such as NMR spectroscopy, have not been applied to directly characterize the linking lysine ε-nitrogen in a PLP-dependent enzyme. NMR chemical shifts are particularly sensitive to local chemical structure; protein and model-compound Schiff base nitrogen atoms show changes in chemical shifts greater than 100 ppm upon protonation.[32] NMR spectroscopy on PLP model compounds by Limbach and co-workers has demonstrated that the tautomeric form favored can depend upon the substituent on the Schiff base nitrogen, solute−solvent interactions, the protonation state of the pyridine nitrogen, and hydrogen bonding to the phenolic oxygen.[32−35] For PLP-dependent enzymes, pyridine nitrogen and select carbon atom chemical shifts have been reported for internal aldimines of alanine racemase and aspartate aminotransferase[12,14] and nitrogen linkages to covalently bound substrates in TS[13] and alanine racemase.[10] However, no chemical shift measurement for a linking Schiff base nitrogen has been reported for any internal aldimine complex. The challenge to interrogating the Schiff base nitrogen is resolution of this single site within the forest of other peaks. Substrate and coenzyme studies rely on the selective introduction of ^{13}C- and ^{15}N-isotopically enriched components to achieve specificity, an approach that is not generally applicable to side-chain sites. Fortunately, the distinct ε-nitrogen chemical shift of a lysine residue covalently bound to the coenzyme can provide resolution of this single site in labeled protein preparations, an approach exploited, for example, to study the Schiff base linkage to retinal in bacteriorhodopsin.[36]

Figure 2 shows ^{15}N solid-state NMR (SSNMR) spectra of catalytically active *S. typhimurium* tryptophan synthase microcrystals prepared under the following four conditions: (A)

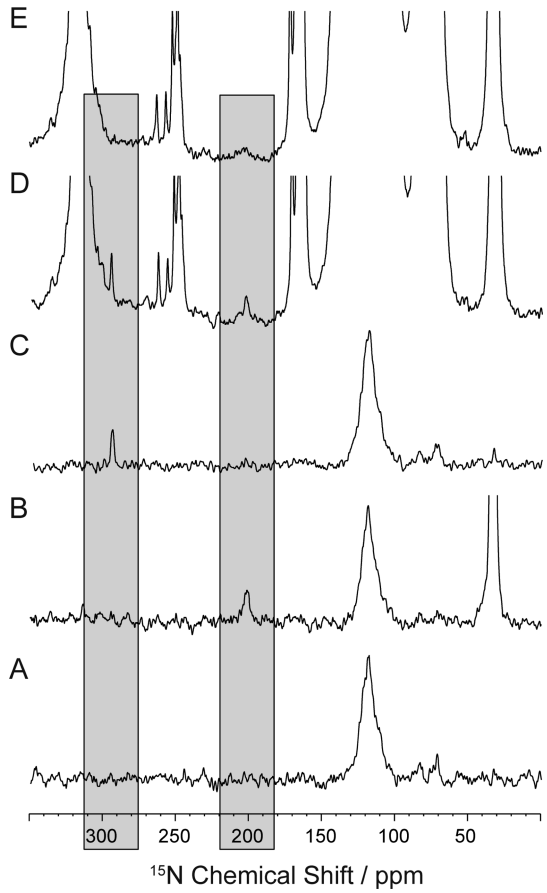

Figure 2. ^{15}N SSNMR cross-polarization magic-angle-spinning (CPMAS) spectra of the tryptophan synthase internal aldimine complex used to assign ^{15}N chemical shifts to the linking Schiff base nitrogen (202.3 ppm) and N1 of PLP (294.7 ppm). Data were acquired on microcrystalline samples of *S. typhimurium* TS prepared with (A) TS at natural-abundance isotopomer concentration, (B) ε-^{15}N-Lys TS, (C) natural-abundance TS/2,2′,3-$^{13}C_3$;^{15}N PLP, and (D, E) U-^{15}N TS/2,2′,3-$^{13}C_3$;^{15}N PLP. (A−C) correspond to direct observation after cross-polarization from ^{1}H to ^{15}N, while (D) and (E) form an ^{15}N{^{13}C}-REDOR pair; both have a 10 ms echo period after cross-polarization, but they differ in the application of π pulses to ^{13}C (at the quarter and three-quarter mark of each rotor period) in (E). Experiments were performed at 9.4 T (400.37 MHz for ^{1}H, 100.69 MHz for ^{13}C, 40.57 MHz for ^{15}N) on a Bruker AVIII spectrometer equipped with an ^{1}H−^{13}C−^{15}N triple-resonance 4 mm MAS probe spinning at a MAS rate of 8 kHz and with the bearing gas cooled to −15 °C, giving an effective sample temperature of −5 °C. Cross-polarization was accomplished at an ^{1}H spin-lock field of 45 kHz, an ^{15}N spin-lock field of 37 kHz (ramped ±2 kHz), and a contact time of 2 ms; 85 kHz Spinal64 ^{1}H decoupling[3] was used throughout. During the REDOR echo periods, 14 μs π pulses were applied to both ^{13}C and ^{15}N. Each spectrum consists of the sum of 81,920 transients acquired with a relaxation delay of 4 s, for a total acquisition time of 3 days 19 h; the REDOR S and S_o spectra were acquired in an interleaved fashion. ^{15}N chemical shifts were referenced indirectly to NH_3(l) via an external solid-state sample of $^{15}NH_4Cl$ calibrated under MAS conditions as described in the SI. For comparison, we found $\delta[NH_3(l)] = \delta[^{15}NH_4Cl(s)] + 39.3$ ppm.

dx.doi.org/10.1021/ja506267d | J. Am. Chem. Soc. 2014, 136, 12824−12827

natural-abundance isotopomer concentration; (B) selectively ^{15}N-enriched at lysine ε-nitrogen sites (ε-^{15}N-Lys TS); (C) selectively ^{13}C/^{15}N-enriched at C2, C2′, C3, and N1 of the PLP coenzyme (TS/2,2′,3-$^{13}C_3$;^{15}N-PLP); and (D, E) uniformly ^{15}N-enriched at protein sites and selectively ^{13}C/^{15}N-enriched at C2, C2′, C3, and N1 of the PLP coenzyme (U-^{15}N-TS/2,2′,3-$^{13}C_3$;^{15}N-PLP). The protocols for the synthesis of 2,2′,3-$^{13}C_3$;^{15}N-PLP (adapted from ref 37) and the preparation of protein samples are given in the Supporting Information (SI). At natural abundance, only signal from the large number of protein backbone nitrogens is observed. Upon incorporation of selectively enriched ε-^{15}N-Lys, two new spectral features are revealed: a large number of mostly overlapped resonances centered at 33 ppm ($\delta[NH_3(l)]$ scale) and a single resonance at 202.3 ppm. The former correspond to charged ε-amino groups on the labeled lysine residues that have been incorporated into the protein, whereas the latter resonates at the position of a protonated Schiff base[32] and is tentatively assigned to N^{ε} of βLys87, the active-site residue that covalently binds PLP. This assignment was confirmed in two ways. First, the Schiff base linkage to βLys87 was broken by addition of the substrate L-serine, which reacts to form an external aldimine intermediate that subsequently loses water to give an aminoacrylate species;[15] upon addition of 5 μL of 1.2 M serine directly to the microcrystalline sample used to obtain the spectrum in Figure 2B, the peak at 202.3 ppm was lost and a new peak at 24.2 ppm appeared, suggestive of a neutral amino lysine side chain for the aminoacrylate intermediate (Figure S1 in the SI). Second, rotational-echo double-resonance (REDOR)[38] experiments were used to specifically edit out (dephase) ^{15}N resonances that are dipolar-coupled to ^{13}C atoms at the 2, 2′, and 3 positions on the PLP ring. As the dipolar coupling falls off as the inverse cube of the interatomic distance, the ^{15}N{^{13}C}-REDOR editing used here (with 10 ms of dipolar dephasing) is selective for nitrogen atoms within ∼3−4 Å of the PLP ^{13}C atoms. The spectra in Figure 2D,E form a REDOR S_o and S pair: both have a 10 ms echo period on ^{15}N before detection, but they differ in the application of dipolar dephasing to ^{13}C for the latter. As expected, there are considerably more peaks in U-^{15}N-TS spectra than in the ε-^{15}N-Lys TS spectrum; there is also remarkable resolution of many individual nitrogen sites. The peak at 202.3 ppm is evident in the REDOR S_o spectrum but is selectively dephased under dipolar couplings to ^{13}C in S. As this peak arises from a lysine ε-nitrogen, we can conclude that this is the resonance of the Schiff base linkage to PLP and, on the basis of its chemical shift,[32] that it is protonated.

Examination of the spectra in Figure 2D,E show a second peak at 294.7 ppm that is also dephased in the REDOR experiment. This peak is not in the spectrum in Figure 2B and thus does not arise from an ε-nitrogen label, but as shown in Figure 2C, it correlates with the introduction of ^{15}N-labeled PLP; this peak is assigned to the PLP pyridine nitrogen (N1). As expected, strong dipolar coupling of N1 to its directly bonded neighbor, C2, leads to efficient REDOR dephasing. This chemical shift of N1 reports that the pyridine nitrogen is deprotonated.[32] Lee−Goldburg cross-polarization,[39] in which the buildup of intensity depends sensitively on the dipolar coupling to hydrogen, confirms the nitrogen protonation states; a short (200 μs) contact time shows appreciable intensity only for the protonated Schiff base nitrogen and not the deprotonated pyridine nitrogen (Figure S2). Additional chemical shift measurements (Figures S3 and S4) for carbons C2 and C3 [158.4 and 168.6 ppm (δ[TMS(l)] scale), respectively] and the phosphorus of the PLP phosphoryl [4.3 ppm ($\delta[H_3PO_4(85\%)]$ scale)] help complete the assignment of ionization states for the coenzyme. For the former, ^{13}C NMR spectroscopy of model Schiff base compounds[40] under conditions in which the ketoenamine form dominates[41] assists in identifying the chemical shifts of C2 and C3 as those for PLP with a deprotonated phenolic oxygen. For the latter, the ^{31}P chemical shift of the phosphoryl definitively reports a dianionic group.[42] The experimentally determined protonation states are shown in Figure 1. We note that neither N1 of PLP nor N^{ε} of βLys87 is at the extreme chemical shift value anticipated for a fully deprotonated or fully protonated nitrogen;[14] this may indicate hydrogen-bonding interactions or equilibria between tautomeric structures that nonetheless strongly favor the form shown.

The ^{15}N SSNMR chemical shift of the Schiff base nitrogen supports the PSB hypothesis for the internal aldimine state of tryptophan synthase. At the same time, ^{13}C, ^{15}N, and ^{31}P chemical shifts on PLP establish that the phosphoryl group, phenolic oxygen, and pyridine ring nitrogen are deprotonated. The pyridine nitrogen in tryptophan synthase interacts with the hydroxyl of βSer377 and, in the absence of an additional proton, would be incapable of assuming the role of hydrogen-bond donor. It has been proposed that the protonation state of the pyridine nitrogen plays an important role in steering later reaction specificity for PLP-dependent enzymes: a protonated pyridine nitrogen enhances electrophilic addition at C4′, while a deprotonated pyridine favors reaction at the substrate C^{α}.[2,7−9,43] The deprotonated pyridine nitrogen in TS, assuming that it is maintained during the catalytic cycle, is consistent with its catalytic role in the β-replacement pathway that takes serine to tryptophan. However, protonation of the PLP nitrogen has been shown to promote proton transfer from the phenolic oxygen to the Schiff base nitrogen,[32,44] a defining aspect of the PSB hypothesis. Effecting this transfer for the unprotonated PLP clearly requires a different mode of activation. Recent work by Toney and Limbach suggests that the formation of the zwitterionic species can be triggered by hydrogen bonding of water molecules to the PLP phenolic oxygen.[2,14] In the active site of TS, there are indeed two crystallographically observed water molecules that form a hydrogen-bonded chain to the phenolic oxygen. Molecular dynamics simulations (detailed in the SI) indicate that the pocket adjacent to the phenolic oxygen contains a network of water molecules that maintain persistent hydrogen-bonding interactions with the phenolic oxygen for the internal aldimine state. The concluded protonation states agree with the proposal that hydrogen bonding of water to the phenolic oxygen is sufficient for activation of catalysis by proton transfer to the Schiff base nitrogen.

The measurement of chemical shifts in the enzyme active site of tryptophan synthase provides a snapshot of protonation states critical for initiating catalysis in this PLP-dependent enzyme. The ^{15}N chemical shift for the linking lysine ε-nitrogen reveals a protonated Schiff base internal aldimine state, while ^{13}C, ^{15}N, and ^{31}P chemical shifts on PLP report that the phosphoryl group, phenolic oxygen, and pyridine ring nitrogen are deprotonated. These results are consistent both with the PSB hypothesis for PLP activation and the role of tryptophan synthase as a β elimination/replacement catalyst. Looking forward, advances in sample preparation, hardware design, and NMR technology [dynamic nuclear polarization (DNP)[45,46] in particular] promise to increase the sensitivity of NMR spectroscopy and allow the measurement of not only isotropic NMR interactions (e.g., chemical shifts) but also anisotropic interactions (e.g., dipolar

and chemical shielding tensors) in functioning enzyme systems such as tryptophan synthase. Along with the current state of the art, these advances will effect an unprecedented, chemically detailed view of the acid–base chemistry that drives catalysis in PLP-dependent enzymes.

■ ASSOCIATED CONTENT

Supporting Information

Additional ^{13}C, ^{15}N, and ^{31}P SSNMR spectra; protocol for preparation of ε-^{15}N-Lys TS and U-^{15}N TS/2,2′,3-^{13}C;^{15}N-PLP; synthesis of 2,2′,3-$^{13}C_3$;^{15}N-PLP; notes on chemical shift referencing; and molecular dynamics simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Dr. Monique Chan-Huot for useful suggestions on the synthesis of isotopically enriched PLP. This work was supported by National Institutes of Health Grant R01GM097569. R.P.Y. and T.J.N. gratefully acknowledge support through NSF GRFP Award DGE-0813967 and U.S. Department of Education GAANN Award P200A120170, respectively.

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