Title
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.

Permalink
https://escholarship.org/uc/item/0sr5s1fn

Journal
Journal of the American Chemical Society, 136(37)

ISSN
0002-7863

Authors
Caulkins, Bethany G
Bastin, Baback
Yang, Chen
et al.

Publication Date
2014-09-03

DOI
10.1021/ja506267d

Peer reviewed
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

Bethany G. Caulkins,† Baback Bastin,‡ Chen Yang,† Thomas J. Neubauer,† Robert P. Young,† Eduardo Hilario,‡ Yu-ming M. Huang,† Chia-en A. Chang,† Li Fan,† Michael F. Dunn,‡ Michael J. Marsella,‡ and Leonard J. Mueller*†

‡Department of Chemistry, University of California, Riverside, California 92521, United States
§Department of Biochemistry, University of California, Riverside, California 92521, United States

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 15N 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 13C, 15N, and 31P 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 B6, acts as a coenzyme in multiple amino acid transformations, including α/β/γ 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,3–6 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 15N 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 13C, 15N, and 31P 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) αβ2/γ 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 transamination reaction in which an amino acid substrate makes a nucleophilic attack at C4′.
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 with PLP-catalyzed reactions in solution.

UV/vis optical spectroscopy of TS supports the PSB hypothesis. Studies of enzyme internal aldime states and model compounds with Schiff base linkages to peptides and amino acids in polar, apotic solvents indicate a conjugation best explained by a protonated Schiff base nitrogen. The internal aldime complexes of most PLP enzymes give absorption maxima in the 420 to 430 nm range, and the 412 nm \( \lambda_{\text{max}} \) 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 \( \pi \) system. The UV/vis spectrum of TS is independent of pH over the range 5.8–10.4, and consequently, there is a single ionic form of the E(Ain) \( \beta \)-site and no change in the distribution of tautomeric structures in this pH range. Deprotonation of PLP enzyme Schiff bases causes shifts to shorter wavelength (~360 nm), while deprotonation of the internal aldime PLP ring nitrogen has only minor effects on the UV/vis spectrum.

The X-ray crystal structures of TS internal aldime complexes show the Schiff base nitrogen in close proximity to the 3'-oxygen of the PLP ring. 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, 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 \( \varepsilon \)-nitrogen in a PLP-dependent enzyme. NMR chemical shifts are particularly sensitive to local chemical environment, and model-compound Schiff base nitrogen atoms show changes in chemical shifts greater than 100 ppm upon protonation. 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. For PLP-dependent enzymes, pyridine nitrogen and select carbon atom chemical shifts have been reported for internal aldimes of alanine racemase and aspartate aminotransferase and nitrogen linkages to covalently bound substrates in TS and alanine racemase. However, no chemical shift measurement for a linking Schiff base nitrogen has been reported for any internal aldime 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 \( ^{13}\text{C} \) and \( ^{15}\text{N} \)-isotopically enriched components to achieve specificity, an approach that is not generally applicable to side-chain sites. Fortunately, the distinct \( \varepsilon \)-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.

Figure 2 shows \( ^{15}\text{N} \) solid-state NMR (SSNMR) spectra of catalytically active S. typhimurium tryptophan synthase microcrystals prepared under the following four conditions: (A)
natural-abundance isotopomer concentration; (B) selectively 15N-enriched at lysine ε-nitrogen sites (ε-15N-Lys TS); (C) selectively 13C/15N-enriched at C2, C2’, C3, and N1 of the PLP coenzyme (TS/2,2’,3-13C,15N-PLP); and (D, E) uniformly 15N-enriched at protein sites and selectively 13C/15N-enriched at C2, C2’, C3, and N1 of the PLP coenzyme (U-15N-TS/2,2’,3-13C,15N-PLP). The protocols for the synthesis of 2,2’,3-13C,15N-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-15N-Lys, two new spectral features are revealed: a large number of mostly overlapped resonances centered at 33 ppm (δ[NH3(1)] 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 resonate at the position of a protonated Schiff base and is tentatively assigned to N’ of β-Lys87, the active-site residue that covalently binds PLP. This assignment was confirmed in two ways. First, the Schiff base linkage to β-Lys87 was broken by addition of the substrate L-serine, which reacts to form an external aldimine intermediate that subsequently loses water to give an aminoaacrylate species, 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 242.2 ppm appeared, suggestive of a neutral amino lysine side chain for the aminoaacrylate intermediate (Figure S1 in the SI). Second, rotational-echo double-resonance (REDOR)38 experiments were used to specifically edit out (dephase) 15N resonances that are dipolar-coupled to 13C 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 15N(1)−13C−15N REDOR editing used here (with 10 ms of dipolar dephasing) is selective for nitrogen atoms within ~3−4 Å of the 13C atoms. The spectra in Figure 2D,E form a REDOR S, and S pair: both have a 10 ms echo period on 14N before detection, but they differ in the application of dipolar dephasing to 13C for the latter. As expected, there are considerably more peaks in U-15N-TS spectra than in the ε-15N-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, spectrum but is selectively dephased under dipolar couplings to 13C 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, 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 15N-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. Lee–Goldburg cross-polarization,30 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(1)] scale), respectively] and the phosphorus of the PLP phosphonyl [4.3 ppm (δ[H3PO4(85%) scale)] help complete the assignment of ionization states for the coenzyme. For the former, 13C NMR spectroscopy of model Schiff base compounds40 under conditions in which the ketoenamine form dominates assists in identifying the chemical shifts of C2 and C3 as those for PLP with a deprotonated phenolic oxygen. For the latter, the 31P 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’ of β-Lys87 is at the extreme chemical shift value anticipated for a fully deprotonated or fully protonated nitrogen; this may indicate hydrogen-bonding interactions or equilibria between tautomeric structures that nonetheless strongly favor the form shown.

The 15N SSNMR chemical shift of the Schiff base nitrogen supports the PSB hypothesis for the internal aldime state of tryptophan synthase. At the same time, 13C, 15N, and 31P 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 C6’.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,52,44 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.2,4 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 aldime 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 15N chemical shift for the linking lysine ε-nitrogen reveals a protonated Schiff base internal aldime state, while 13C, 15N, and 31P 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

dx.doi.org/10.1021/ja506267d/jacs.136,12824–12827
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**

2 Supporting Information

Additional $^{13}$C, $^{15}$N, and $^{31}$P SSNMR spectra; protocol for preparation of $\varepsilon$-$^{15}$N-Lys TS and U-$^{15}$N TS/2,2-$^{13}$C, $^{15}$N-PLP; synthesis of 2,2-$^3$-$^{13}$C, $^{15}$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.

**AUTHOR INFORMATION**

Corresponding Author

leonard.mueller@ucr.edu

**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.

**REFERENCES**

(1) Metzler, D. E.; Ikawa, M.; Snell, E. E. J. Am. Chem. Soc. 1954, 76, 648.
(2) Toney, M. D. Biochim. Biophys. Acta 2011, 1814, 1407.
(3) Fung, B. M.; Khitrin, A. K.; Ermolaev, K. J. Magn. Reson. 2000, 142, 97.
(4) Cordes, E. H.; Jencks, W. P. Biochemistry 1962, 1, 773.
(5) Heinert, D.; Martell, A. E. J. Am. Chem. Soc. 1963, 85, 188.
(6) Metzler, D. E. J. Am. Chem. Soc. 1957, 79, 485.
(7) Cruegeras, J.; Rios, A.; Riveros, E.; Richard, J. P. J. Am. Chem. Soc. 2011, 133, 3173.
(8) Major, D. T.; Gao, J. J. Am. Chem. Soc. 2006, 128, 16345.
(9) Major, D. T.; Nam, K.; Gao, J. L. J. Am. Chem. Soc. 2006, 128, 8114.
(10) Copie, V.; Faraci, W. S.; Walsh, C. T.; Griffin, R. G. Biochemistry 1988, 27, 4966.
(11) McDowell, L. M.; Lee, M. S.; Schaefer, J.; Anderson, K. S. J. Am. Chem. Soc. 1995, 117, 12352.
(12) Sharif, S.; Fogle, E.; Toney, M. D.; Denisov, G. S.; Shenderovich, I. G.; Buntkowsky, G.; Tolstoy, P. M.; Huot, M. C.; Limbach, H. H. J. Am. Chem. Soc. 2007, 129, 9358.
(13) Lai, J. F.; Nisk, D.; Wang, Y. C.; Domratcheva, T.; Barends, T. R. M.; Schwarz, F.; Olsen, R. A.; Elliott, D. W.; Fatmi, M. Q.; Chang, C. E. A.; Schlichting, I.; Dunn, M. F.; Mueller, L. J. J. Am. Chem. Soc. 2011, 133, 4.
(14) Chan-Huot, M.; Dos, A.; Zander, R.; Sharif, S.; Tolstoy, P. M.; Compton, S.; Fogle, E.; Toney, M. D.; Shenderovich, I.; Denisov, G. S.; Limbach, H. H. J. Am. Chem. Soc. 2013, 135, 18160.
(15) Dunn, M. F.; Nisk, D.; Ngo, H.; Barends, T. R. M.; Schlichting, I. Trends Biochem. Sci. 2008, 33, 254.
(16) Yanofsky, C.; Crawford, I. P. In The Enzymes; Boyer, P. D., Ed.; Academic Press: New York, 1972; p 1.
(17) Miles, E. W. Adv. Enzymol. Relat. Areas Mol. Biol. 1979, 49, 127.
(18) Lane, A. N.; Kirschner, K. Eur. J. Biochem. 1983, 129, 571.
(19) Christensen, H. N. J. Am. Chem. Soc. 1958, 80, 99.
(20) Peracchi, A.; Bettati, S.; Mozzarelli, A.; Rossi, G. L.; Miles, E. W.; Dunn, M. F. Biochemistry 1996, 35, 1872.
(21) Goldberg, M. E.; Baldwin, R. L. Biochemistry 1967, 6, 2113.
(22) Hur, O.; Nisk, D.; Casino, P.; Dunn, M. F. Biochemistry 2002, 41, 9991.
(23) Bazhulina, N. P.; Morozov, Y. V.; Papisova, M. I.; Demidkina, T. V. Eur. J. Biochem. 2000, 267, 1830.
(24) Christen, P.; Metzler, D. E. Transaminases; Wiley: New York, 1998.
(25) Metzler, C. M.; Cahill, A.; Metzler, D. E. J. Am. Chem. Soc. 1980, 102, 6075.
(26) Hyde, C. C.; Ahmed, S. A.; Padlan, E. A.; Miles, E. W.; Davies, D. R. J. Biol. Chem. 1988, 263, 17857.
(27) Sachpatzidis, A.; Dealwis, C.; Lubetsky, J. B.; Liang, P. H.; Anderson, K. S.; Lolis, E. Biochemistry 1999, 38, 12665.
(28) Kulik, V.; Weyand, M.; Seidel, R.; Nisk, D.; Arcas, D.; Dunn, M. F.; Schlichting, I. J. Mol. Biol. 2002, 324, 677.
(29) Weyand, M.; Schlichting, I.; Marabotti, A.; Mozzarelli, A. J. Biol. Chem. 2002, 277, 10647.
(30) Ngo, H.; Harris, R.; Kimmich, N.; Casino, P.; Nisk, D.; Blumenstein, L.; Barends, T. R.; Kulik, V.; Weyand, M.; Schlichting, I.; Dunn, M. F. Biochemistry 2007, 46, 7713.
(31) Nisk, D.; Hilario, E.; Dierkers, A.; Ngo, H.; Borchardt, D.; Neubauer, T. J.; Fan, L.; Mueller, L. J.; Dunn, M. F. Biochemistry 2013, 52, 6396.
(32) Limbach, H. H.; Chan-Huot, M.; Sharif, S.; Tolstoy, P. M.; Shenderovich, I. G.; Denisov, G. S.; Toney, M. D. Biochim. Biophysics Acta 2011, 1814, 1426.
(33) Chan-Huot, M.; Sharif, S.; Tolstoy, P. M.; Toney, M. D.; Limbach, H. H. Biochemistry 2010, 49, 10818.
(34) Sharif, S.; Denisov, G. S.; Toney, M. D.; Limbach, H. H. J. Am. Chem. Soc. 2007, 129, 6313.
(35) Sharif, S.; Schagen, D.; Toney, M. D.; Limbach, H. H. J. Am. Chem. Soc. 2007, 129, 4440.
(36) Harbison, G. S.; Herzfeld, J.; Griffin, R. G. Biochemistry 1983, 22, 1.
(37) Florentiev, V. L.; Ivanov, V. I.; Karpeisky, M. Y. Methods Enzymol. 1970, 18A, 567.
(38) Guillou, T.; Schaefer, J. J. Magn. Reson. 1989, 81, 196.
(39) van Rossum, B. J.; de Groot, C. P.; Ladhizhansky, V.; Vega, S.; de Groot, H. J. M. J. Am. Chem. Soc. 2000, 122, 3465.
(40) O’Leary, M. H.; Payne, J. R. J. Biol. Chem. 1976, 251, 2248.
(41) Sharif, S.; Huot, M. C.; Tolstoy, P. M.; Toney, M. D.; Jonsson, K. H. M.; Limbach, H. H. J. Phys. Chem. B 2007, 111, 3869.
(42) Schnackez, K. D.; Andi, B.; Cook, P. F. Biochim. Biophys. Acta 2011, 1814, 1447.
(43) Casasnovas, R.; Adrover, M.; Ortega-Castro, J.; Frau, J.; Donoso, J.; Munoz, F. J. Phys. Chem. B 2012, 116, 10665.
(44) Sharif, S.; Powell, D. R.; Schagen, D.; Steiner, T.; Toney, M. D.; Fogle, E.; Limbach, H. H. Acta Crystallogr., Sect. B 2006, 62, 480.
(45) Hall, D. A.; Maus, D. C.; Gerfen, G. J.; Inati, S. J.; Becerra, L. R.; Dahlquist, F. W.; Griffin, R. G. Science 1997, 276, 930.
(46) Bajaj, V. S.; Mak-Jurkauskas, M. L.; Belenyk, M.; Herzfeld, J.; Griffin, R. G. Proc. Natl. Acad. Sci. U.S.A. 2009, 106, 9244.