Evidence for Conformational Movement and Radical Mechanism in the Reaction of 4-Thia-L-lysine with Lysine 5,6-Aminomutase

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · SEPTEMBER 2009

Impact Factor: 3.3 · DOI: 10.1021/jp905357a · Source: PubMed

THE JOURNAL OF PHYSICAL CHEMISTRY B LETTERS

2009, *113,* 12161–12163
Published on Web 08/17/2009

Evidence for Conformational Movement and Radical Mechanism in the Reaction of 4-Thia-L-lysine with Lysine 5,6-Aminomutase

Amarendra N. Maity,[†] Chih-Pin Hsieh,[†] Ming-Hui Huang,[†] Yung-Han Chen,[†] Kuo-Hsiang Tang,[‡,§] Elham Behshad,[‡,∥] Perry A. Frey,[‡] and Shyue-Chu Ke*,[†]

Physics Department, National Dong Hwa University, Hualien, Taiwan 974-01, Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53726

Received: June 8, 2009; Revised Manuscript Received: July 29, 2009

We demonstrate that the steady state reaction of lysine 5,6-aminomutase with substrate analogue 4-thia-L-lysine generates a radical intermediate, which accumulates in the enzyme to an electron paramagnetic resonance (EPR) detectable level. EPR line width narrowing of ∼1 mT due to [4′-^{2}H] labeling of the pyridoxal-5′-phosphate (PLP), an isotropic hyperfine coupling of 40 MHz for the proton at C4′ of PLP derived from ^{2}H electron nuclear double resonance (ENDOR) measurement, and spin density delocalization onto the ^{31}P of PLP realized from observations of the ^{31}P ENDOR signal provide unequivocal identification of the radical as a substrate−PLP-based species. X- and Q-band EPR spectra fittings demonstrate that this radical is spin coupled with the low spin Co^{2+} in cob(II)alamin and the distance between the two species is about 10 Å. These results provide direct evidence for the active site motion upon substrate binding, bringing the adenosylcobalamin to the proximity of substrate−PLP for subsequent H-atom abstraction and for the notion that lysine 5,6-aminomutase functions by a radical mechanism. Observation of ^{2}H-ENDOR signal also provides a reliable hyperfine coupling constant for future comparison with quantum-mechanical-based calculations to gain further insight into the molecular structure of this steady state radical intermediate.

Introduction

Lysine 5,6-aminomutase (5,6-LAM) is essential for the metabolism of L-lysine and D-lysine in anaerobic bacteria, many species of which are pathogenic, e.g., *Porphyromonas gingivalis*, *Clostridium difficile*, etc.[1] 5,6-LAM is an adenosylcobalamin (AdoCbl) and pyridoxal-5′-phosphate (PLP) dependent enzyme that catalyzes reversible 1,2-shift of the ε-amino group of DL-lysine or L-β-lysine into 2,5- or 3,5-diaminohexanoic acid.[2] PLP is believed to form a substrate−PLP covalent complex through an aldimine linkage during catalytic turnover, and the role of AdoCbl is to provide a source of 5′-deoxyadenosyl radical (Ado•) by homolytic cleavage of the Co−C bond; both steps are essential in the mechanism of action. The radical catalysis (Scheme 1) is thought to begin with the initiator Ado• abstracting a H-atom from the substrate−PLP complex, forming an activated substrate−PLP radical (S•), which is presumed to rearrange through a cyclic aziridinylcarbinyl−PLP radical (I•) to the product−PLP radical (P•). The P• reabstracts a H-atom from Ado-H to form the product. This proposed mechanism[3] is analogous to that of the adenosylmethionine (AdoMet) and PLP dependent L-lysine 2,3-aminomutase (2,3-LAM)[4] which catalyzes a similar 1,2-shift of an amino group. For 2,3-LAM, Frey, Reed, and co-workers have provided thorough evidence for the S• of the 4-thialysine analogue,[4a] the P• of β-lysine,[4b] and an allylic analogue of Ado•.[5] Absent, however, has been a direct observation of the elusive I• linking the S• and P•.

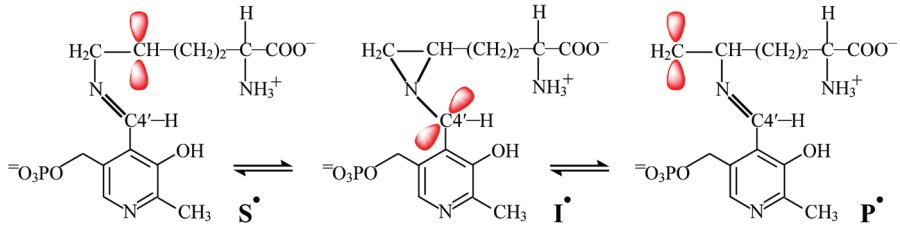
Understanding the radical mediated covalent bond catalysis, with the help of electron paramagnetic resonance (EPR) spectroscopy, has a venerable history in AdoMet and AdoCbl dependent biochemistry.[6] However, for 5,6-LAM, no radical intermediate has been observed to support the proposed radical mechanism, presumably because the radicals are not stable enough to detectably accumulate during turnover on natural substrates. As we anticipate that 4-thia-L-lysine (*S*-2-aminoethyl-L-cysteine) might react with 5,6-LAM in place of L-lysine, the possibility of exploiting accumulated sulfur stabilized radical intermediates in elucidating the mechanism of action of this enzyme should be urgently investigated. Here, we present spectroscopic evidence for the participation of a radical intermediate in 5,6-LAM by using 4-thia-L-lysine as an alternative substrate, [4′-^{2}H] labeled PLP, EPR, and electron nuclear double resonance (ENDOR) spectroscopies.

The recombinant 5,6-LAM from *Clostridium sticklandii* was produced by expression in *Escherichia coli* and purified following the procedures described by Chang and Frey[2] with the exception that PLP was omitted to the lysis and purification buffers. [4′-^{2}H]PLP was synthesized following the literature procedure for [4′-^{3}H]PLP.[7] EPR data were collected using a Bruker EMX spectrometer. The EPR spectra were acquired from single scans. ENDOR spectra were recorded with a Bruker DICE ENDOR assembly equipped with a high power RF amplifier (ENI 3200 L) to generate the CW B_2 field in the cavity. ENDOR spectra were collected in frequency modulation mode. The

* To whom correspondence should be addressed. E-mail: ke@mail.ndhu.edu.tw. Phone: +886-3863-3705.
† National Dong Hwa University.
‡ University of Wisconsin—Madison.
§ Current address: Departments of Biology and Chemistry, Washington University, St. Louis, MO 63130.
∥ Current address: Incyte Corporation, Wilmington, DE 19809.

10.1021/jp905357a CCC: $40.75

SCHEME 1: Proposed PLP-Bound Radical Intermediates in the Reaction of D-Lysine with 5,6-LAM



ENDOR spectra were the average of 300 scans. The instrument settings are shown in the figure legend. For EPR sample preparation, 5,6-LAM (0.3 mM) was incubated with 100 mM NH_4EPPS buffer (pH 8.5), 5 mM dithiothreitol, 0.3 mM adenosylcobalamin, and 0.3 mM PLP at 37 °C for 3 min. Reaction was initiated by addition of 30 mM 4-thia-L-lysine to the preincubated enzymatic system. The reaction mixture (~0.3 mL) was allowed to proceed for 2 min and then transferred to an EPR tube and frozen in liquid nitrogen chilled isopentane. All manipulations were carried out aerobically under red light. Identical EPR signals were obtained for samples prepared anaerobically. *EasySpin* was employed to simulate the EPR and ENDOR spectra.[8a]

The X-band EPR spectrum of the radical intermediate generated in the steady state reaction of 5,6-LAM with 4-thia-L-lysine is shown in Figure 1a. Repeated measurements of different samples indicated that the two outer broad transitions are not due to baseline drift but are part of a general four-line pattern for a weakly coupled biradical system, wherein the two paramagnets retain separate EPR identities and the outer transitions are split from the corresponding inner ones by approximately exchange coupling constant, J. The overall appearance of the X-band EPR spectrum thus suggests the presence of a cob(II)alamin species interacting with an organic radical in the active site of 5,6-LAM. The spectrum of the radical intermediate was further acquired at the Q-band (Figure 1b) to provide a stringent test for spectral simulation (see the Supporting Information for details).

The EPR spectra were simulated using a biradical spin-Hamiltonian that includes one-electron spin-Hamiltonians, isotropic exchange J, zero field splitting D, and nuclear hyperfine interaction of low spin Co^{2+} with the ^{59}Co nuclear spin ($I = {}^7/_2$).[8a] Other nuclear hyperfine interactions are assumed to contribute only to the inhomogeneous line width of the EPR features and are neglected in the simulation. Eulerian angles were used to relate the coordinate system and the principal g-axis system of the organic radical to the principal g-axis of Co^{2+}. A satisfactory concurrent fit to both X- and Q-band spectra is shown in Figure 1. If we assume that the dependence of the zero field splitting parameter D (G) on the distance R (Å) between two paramagnets is solely due to point dipole−dipole interaction with $D = 6.95 \times 10^3\, g_1 g_2 / R^3$,[8b] we obtain a distance R of 10.6 Å between Co^{2+} and the organic radical. This distance estimate is strengthened by employing the empirical limit function,[8c] $|J| \leq 1.35 \times 10^7\, e^{-1.8R}$, which leads to a distance of $R \leq 10.8$ Å. EPR study thus provides direct evidence for the hypothesis derived from analysis of the precatalytic, substrate-free crystal structure,[9] suggesting that a large scale conformational change of the enzyme upon substrate binding is required to bring the AdoCbl initially placed 25 Å away from the active site to the proximity of substrate−PLP for subsequent H-atom abstraction and radical catalysis.

Following the strategy developed in the study of 2,3-LAM,[4a] we compare the line width of the main feature in the X-band EPR spectrum of the [4′-^{2}H]PLP labeled sample (see Scheme 1 for atom labeling) with that of the unlabeled sample (Figure 1a, bottom left). Unlike the case of 2,3-LAM wherein the two EPR spectra were virtually identical,[4a,10] the ~1 mT line width narrowing in the labeled sample relative to the unlabeled sample in 5,6-LAM ($\gamma_{1H}/\gamma_{2H} = 6.51$) immediately suggests that the radical is a substrate−PLP-based radical and the unpaired electron spin is strongly coupled to the ^{2}H at the 4′-position of PLP. ENDOR spectra (Figure 2) were collected at 3315 G corresponding to the maximum absorption of the substrate−PLP-based radical EPR signal wherein all directions of space

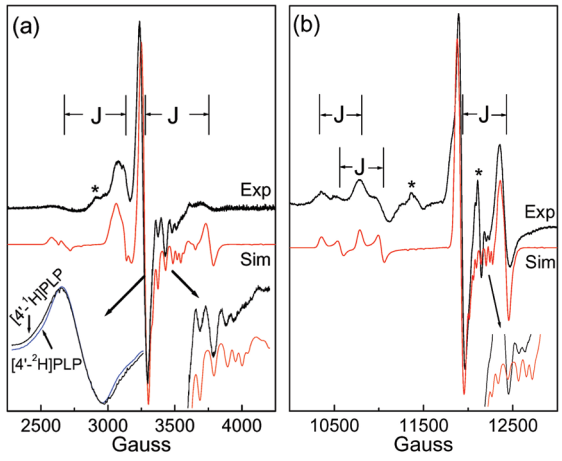


Figure 1. (a) X-band and (b) Q-band EPR spectra of the radical intermediate in 5,6-LAM reaction with 4-thia-L-lysine. Experimental: microwave frequency, (a) 9.535, (b) 33.96 GHz; power, (a) 2, (b) 1.46 mW; modulation, 8 G at 100 kHz; $T = 80$ K. The starred feature in part a is the g_{xy} signal of free Co^{2+}, and those in part b are contaminants present in variable amounts in the samples. Simulation: $g_{Co} = [2.285\ 2.236\ 2.01]$, $g_{radical} = [2.01\ 2.0\ 1.995]$, Euler angle = [60° 10° 120°]; $A_{Co} = [8\ 5\ 112]$ G; $J = 500$ G, $D = -25$ G, $E = 0$, Euler angle = [0° 80° 0°]; line width = [40 40 20] G.

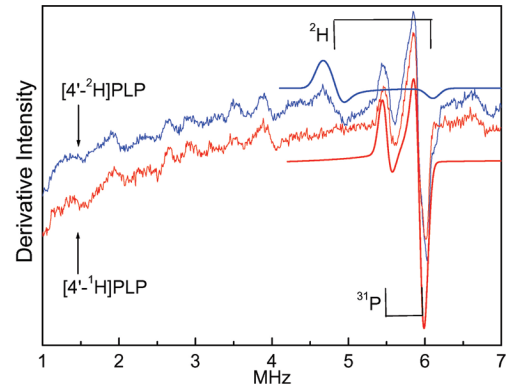


Figure 2. X-band ENDOR spectra of the radical intermediate in 5,6-LAM reaction with 4-thia-L-lysine. Conditions: $B = 3315$ G; $\nu = 9.595$ GHz; microwave power, 2 mW; RF power, 200 W; modulation depth, 100 kHz; $T = 20$ K. Simulation parameters: $g_{2H} = 0.857$; $g_{31P} = 2.263$; $A_{2H} = [-5, -5.4, -7.9]$ MHz; $A_{31P} = [-0.4, -0.5, 19.2]$ MHz; line width, 150 kHz.

contribute to the EPR envelope. The spectrum of the sample containing [4′-^{2}H]PLP (upper trace) exhibits two additional features near 4.8 and 6.1 MHz that are assigned to ^{2}H by virtue of the absence in the spectrum of the unlabeled sample (lower trace). The matching low frequency signals for the two peaks, expected to mirror about half the hyperfine coupling and split by twice the free ^{2}H Larmor frequency (2.16 MHz), occurring at ~0.5 and ~1.8 MHz were not observed with the Bruker X-band DICE ENDOR accessory, but this is not unexpected. If we neglect the ^{2}H nuclear quadrupole contribution (normally <0.5 MHz) and assign the broad derivative feature near 4.8 MHz and the negative peak at 6.1 MHz to perpendicular and parallel transitions, respectively, then a value of $|A_{iso}(^2H)| \sim 6$ MHz is derived, which corresponds to $|A_{iso}(^1H)| \sim 40$ MHz.

This value is comparable either with a α-proton coupling of $>C_\alpha H$ fragment corresponding to a significant amount of unpaired spin density ($\rho_\pi^\alpha = 0.57$) on the C4′-position of PLP or with a hyperconjugative β-proton coupling of $N_\alpha C4'_\beta H_\beta$ type in which significant spin densities reside on the migrating N. Although an unequivocal structure description for the observed radical intermediate cannot be settled on the basis of the available data, direct observation of the ^{2}H ENDOR signal provides a reliable hyperfine coupling constant for future comparison with quantum-mechanical-based calculations to gain further insight into the molecular structure of this radical intermediate.

In Figure 2, both ENDOR spectra also show a pair of lines at 5.54 and 5.90 MHz mirrored about the Larmor precession frequency of ^{31}P (5.72 MHz); they are assigned to hyperfine coupling with ^{31}P of the PLP on the basis of the following three facts. First, the sample has never been exposed to phosphate buffer during preparation. Second, these signals cannot be observed in the region where the Co^{2+} EPR signal occurs. Third, AdoCbl binds to this enzyme in the base-off mode, which moves the phosphodiester away from the corrin ring. This and the intervening saturated covalent bonds apparently remove the phosphodiester from the spin system. A model dominated by weak isotropic hyperfine coupling (~0.36 MHz) cannot account for the observed asymmetrical line shapes. Alternatively, the two derivative shaped features are interpreted as the perpendicular singularities of a dipolar powder pattern. Although the asymmetric line shape is fit well (Figure 2), a distance estimate will not be quantitatively accurate due to violations of the point dipole approximation and likely non-negligible local dipolar interaction with the spin density in the P 3p orbital. However, the data are clearly sufficient to conclude that the radical intermediate is a conjugated system, from which spin density covalently transferred from the radical center to ^{31}P of the PLP can be realized. Such spin delocalization phenomena would help to stabilize the radical intermediate to a detectable concentration.

In summary, three early steps in the reaction of 4-thia-L-lysine with 5,6-LAM can be construed on the basis of the data presented here. First, rather than a motionlessly binding of the substrate, EPR data and simulation demonstrate that transaldimination of the lysine-144 linkage brings the AdoCbl to the proximity of substrate and PLP and triggers homolysis of AdoCbl and the formation of 5′-Ado•. Second, EPR line width narrowing due to [4′-^{2}H] labeling of PLP and direct ^{2}H ENDOR measurement demonstrate that the 5′-Ado• abstracts a H-atom from the substrate−PLP complex, initiating radical catalysis. Third, ^{2}H and ^{31}P ENDOR data together indicate that 4-thia-L-lysine is linked covalently to the cofactor PLP as significant scalar couplings were observed, requiring a through-bond connection between the unpaired electron and the cofactor PLP. These steps mimic those early steps in the reaction mechanism expected for reaction with natural substrates, providing direct proof for earlier proposition of active site motion upon substrate binding and support to the notion that 5,6-LAM functions by a radical mechanism.

Acknowledgment. This research was supported by the National Science Council Grant NSC97-2627-M-259-001, Taiwan (to S.-C.K.), and the US National Institute of Diabetes and Digestive and Kidney Diseases Grant DK28607 (to P.A.F.).

Supporting Information Available: Analysis of EPR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) Stadtman, T. C. *Adv. Enzymol.* **1973**, *38*, 413–447. (b) Baker, J. J.; Stadtman, T. C. In *B_{12}*; Dolphin, D., Ed.; John Wiley & Sons: New York, 1982; Vol. 2, pp 203−232.

(2) Chang, C. H.; Frey, P. A. *J. Biol. Chem.* **2000**, *275*, 106–114.

(3) (a) Tang, K. H.; Chang, C. H.; Frey, P. A. *Biochemistry* **2001**, *40*, 5190–5199. (b) Tang, K. H.; Harms, A.; Frey, P. A. *Biochemistry* **2002**, *41*, 8767–8776. (c) Tang, K. H.; Casarez, A. D.; Wu, W.; Frey, P. A. *Arch. Biochem. Biophys.* **2003**, *418*, 49–54.

(4) (a) Wu, W.; Lieder, K. F.; Reed, G. H.; Frey, P. A. *Biochemistry* **1995**, *34*, 10532–10537. (b) Ballinger, M. D.; Frey, P. A.; Reed, G. H. *Biochemistry* **1992**, *31*, 10782–10789. (c) Lees, N. S.; Chen, D.; Walsby, C. J.; Behshad, E.; Frey, P. A.; Hoffman, B. M. *J. Am. Chem. Soc.* **2006**, *128*, 10145–10154. (d) Ballinger, M. D.; Frey, P. A.; Reed, G. H. *Biochemistry* **1995**, *34*, 10086–10093.

(5) (a) Magnusson, O. T.; Reed, G. A.; Frey, P. A. *J. Am. Chem. Soc.* **1999**, *121*, 9764–9765. (b) Magnusson, O. T.; Reed, G. A.; Frey, P. A. *Biochemistry* **2001**, *40*, 7773–7782.

(6) (a) Banerjee, R. *Chemistry and Biochemistry of B12*; Wiley: New York, 1999. (b) Banerjee, R.; Ragsdale, S. W. *Annu. Rev. Biochem.* **2003**, *72*, 209–247. (c) Frey, P. A.; Magnusson, O. T. *Chem. Rev.* **2003**, *103*, 2095–2127. (d) Frey, P. A.; Hegeman, A. D.; Reed, G. H. *Chem. Rev.* **2006**, *106*, 3302–3316. (e) Reed, G. H.; Mansoorabadi, S. O. *Curr. Opin. Struct. Biol.* **2003**, *13*, 716–721.

(7) Fu, M.; Silverman, R. B. *Bioorg. Med. Chem.* **1999**, *7*, 1581–1590.

(8) (a) Stoll, S.; Schweiger, A. *J. Magn. Reson.* **2006**, *178*, 42–55. (b) Coffman, R. E.; Buettner, G. R. *J. Phys. Chem.* **1979**, *83*, 2387–2392. (c) Luckhurst, G. R. In *Spin Labeling: Theory and Applications*; Berliner, L. J., Ed.; Academic Press: New York, 1976.

(9) Berkovitch, F.; Behshad, E.; Tang, K.-H.; Enns, E. A.; Frey, P. A.; Drennan, C. L. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15870–15875.

(10) We note that the electron spin echo envelope modulation technique was required to probe the weak hyperfine interaction between the α-radical of β-lysine and the ^{2}H at the 4′-position of PLP; $A_{iso}(^2H) = 0.1$ MHz.

JP905357A