J|A|C|S
COMMUNICATIONS

Published on Web 04/23/2004

Direct Determination of Product Radical Structure Reveals the Radical Rearrangement Pathway in a Coenzyme B_{12}-Dependent Enzyme

Kurt Warncke* and Jeffrey M. Canfield

Department of Physics, Emory University, Atlanta, Georgia 30322-2430

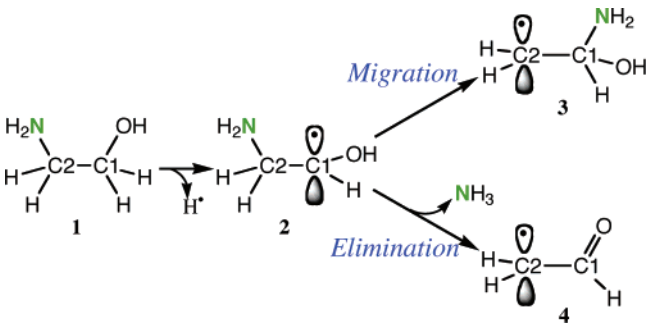
Received December 7, 2003; E-mail: kwarncke@physics.emory.edu

Coenzyme B_{12}-dependent enzymes use radical intermediates to catalyze 1,2-shifts of carbon (Class I, carbon skeleton mutases), heteroatoms (Class II eliminases, including ethanolamine deaminase), and amino groups (Class III, aminomutases).[1−3] The elucidation of the rearrangement mechanisms and how atom displacements are guided by the protein have been long-standing issues in the B_{12} enzyme field. Experimental approaches (isotope exchange, X-ray crystallography of stable (diamagnetic) enzyme states, and mutagenesis in combination with thermodynamic/kinetic studies),[1,2] along with ab initio calculations on reaction models,[4,5] have provided insights into the reaction mechanisms. Absent, however, has been a specification of a rearrangement mechanism based on the direct determination of radical intermediate structure. The bacterial ethanolamine deaminase[3,6] currently offers an opportunity for a direct analysis, since the cryotrapped substrate radical state[7] has been recently well-characterized by high-resolution electron paramagnetic resonance (EPR) techniques,[8−11] and the cryotrapped product radical state is the only example reported in a coenzyme B_{12}-dependent enzyme.[12] Here, we present the first direct spectroscopic determination of the structure of the product radical in ethanolamine deaminase from *Salmonella typhimurium*, by using isotopic substitution and EPR spectroscopies. The results reveal the pathway of the radical rearrangement.

Radical catalysis in ethanolamine deaminase is initiated by homolysis of the coenzyme cobalt−carbon bond, producing the 5′-deoxyadenosyl-C5′ radical center that subsequently abstracts a hydrogen atom from C1 of the substrate aminoethanol **1** to produce the substrate radical **2** (Scheme 1).[1,2] Rearrangement of the substrate radical to a C2-centered product radical has been proposed to proceed along two possible pathways:[6] (a) The amine migration pathway[13] that leads to a carbinolamine radical **3** and (b) the amine elimination pathway that leads to the acetaldehyde radical **4** and ammonia. In reactions of 1,2-aminoalkanols in solution, reaction proceeds by the amine elimination pathway with corresponding aldehyde radical intermediates.[14] However, it has been proposed, by analogy with the rearrangement in the Class II diol dehydrase[15] and confinement of reacting species in the active site,[16] that the amine migration pathway is followed on the enzyme.

As shown in Scheme 1, the presence of covalently bonded substrate nitrogen in the product radical distinguishes the migration and elimination pathways. A difference between the EPR line shapes of product radicals generated with ^{14}N- or ^{15}N-labeled aminoethanol would be indicative of a covalently bonded nitrogen atom, because of the different electron−nuclear hyperfine coupling with the magnetically distinct ^{14}N (nuclear spin $I = 1$, nuclear g-value $g_N = 0.4038$) and ^{15}N ($I = 1/2$, $g_N = -0.5664$). Nearby, but noncovalently bonded, nitrogen is not detected in the EPR spectrum.[17] Figure 1A shows the EPR spectra of ^{14}N- and ^{15}N-labeled product radical samples, which were generated under identical conditions by cryotrapping during steady-state turnover.

Scheme 1



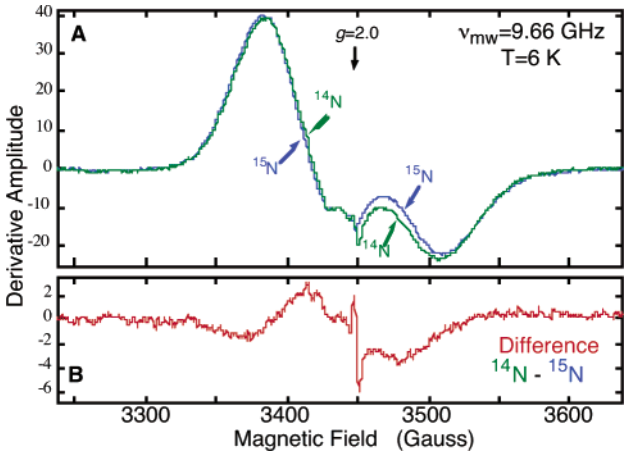


Figure 1. EPR spectra of the ^{14}N- and ^{15}N-labeled product radical in ethanolamine deaminase (A) and the ^{14}N−^{15}N difference spectrum (B).

As shown by the EPR line shapes in Figure 1A and the ^{14}N−^{15}N difference spectrum in Figure 1B, the line shape is sensitive to nitrogen isotope. Results from three separate ^{14}N,^{15}N sample preparations reproduce the same difference line shape (Figure S1, Supporting Information). ^{14}N−^{15}N substitution primarily alters spectral amplitudes in the interior of the line shape. This is in agreement with resolution-enhancement analysis of ^{14}N−^{15}N differences in *substrate* radical spectra, which show little change in line width but substantial changes in interior line shape.[11] The observed modest ^{14}N−^{15}N line shape effects are expected from previous ^{2}H−^{1}H and ^{13}C−^{12}C isotope substitution studies.[7,12] This is because the unresolved hyperfine coupling contributions are dominated by line-broadening because of relaxation by the nearby paramagnetic Co^{II} and anisotropy in the Co^{II}-radical electron−electron interaction. On the basis of these results, we propose the carbinolamine structure **3** for the product radical and, therefore, that the radical rearrangement proceeds by the amine migration pathway in ethanolamine deaminase. The results do not distinguish between dissociative (fragmentation−recombination) or associative (concerted) mechanisms for amine migration. The departure of the

10.1021/ja031569d CCC: $27.50

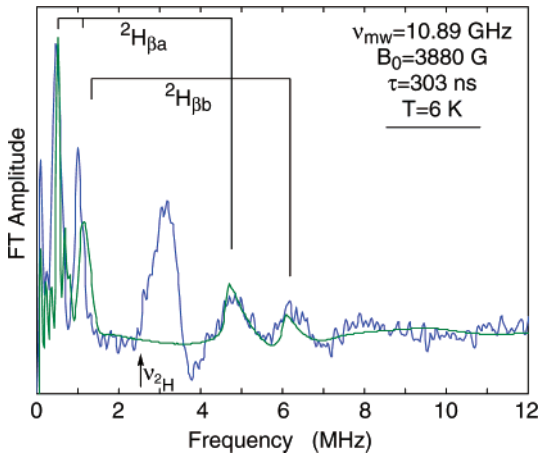


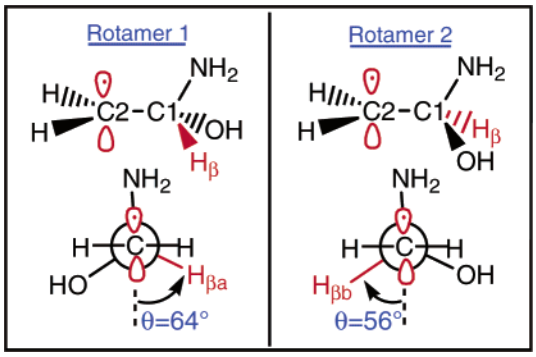
Figure 2. Fourier transform of three-pulse $^2H/^1H$ quotient ESEEM from the product radical intermediate in ethanolamine deaminase and overlaid simulation (green) of β-^2H features.

enzyme reaction from the solution elimination pathway appears to reflect avoidance of the acetaldehyde radical, which calculations indicate[5] is not thermodynamically competent for hydrogen atom reabstraction from the C5′ methyl group.

The product radical structure was further studied by using three-pulse electron spin−echo envelope modulation (ESEEM) spectroscopy of electron ^2H coupling in the product radical generated by using 1,1,2,2-$^2H_4$-labeled and natural abundance ($^1H_4$-) aminoethanol. The ESEEM frequency spectrum presented in Figure 2 shows a multicomponent line shape composed of features from ^2H incorporated into the C5′ methyl group (2.5−3.7 MHz) and two pairs of features that are assigned to two classes of β-^2H ($^2H_{\beta a}$, $^2H_{\beta b}$), present in approximately equal proportions, based on the parameters for the overlaid simulation (Table S1). A full simulation analysis of the product radical ^2H ESEEM will be presented separately (K.W., in preparation). The two β-^2H couplings are assigned to two different C1−C2 rotamer states of the product radical.

The Heller−McConnell expression, $A_{iso}=\rho B_2 \cos^2 \theta$,[18] relates the isotropic hyperfine coupling (A_{iso}) for the β-H interaction to the dihedral angle, θ, between the C1−H_β bond and p-orbital axis (B_2 = 24.9 MHz for ^2H)[19] and the unpaired electron spin density at the coupling atom (C2), $\rho = 1$. Substitution of the $^2H_{\beta a}$ and $^2H_{\beta b}$ A_{iso} values of 4.9 and 7.8 MHz into the Heller−McConnell expression leads to calculated dihedral angles of 64° and 56°, respectively. The sum of these dihedral angles is 120°, which is equal to the angular difference between substituents on an sp^3-hybridized carbon atom. These results are accounted for in the model presented in Chart 1 by orienting the two C1−C2 rotamers so that $^2H_{\beta a}$ and $^2H_{\beta b}$ are positioned on opposite sides of the C1−C2 axis. The model gives primacy to strong amino group−protein interaction(s) in determining the in situ conformational state of the product radical. Proposals for H−C1−O reorientation mechanisms are given in the Supporting Information (Figure S2).

Chart 1



The essentially coparallel orientation of the C1−N bond with the partially filled p-orbital on C2 in the product radical is the same as the [C2−N] versus [C1 p-orbital] orientation inferred for the substrate radical.[6,11] This common orbital alignment motif in the two radical rearrangement intermediates provides additional support for the amine migration mechanism and leads to the prediction of specific protein hydrogen-bonding groups that anchor, and thus define, the stereospecific in situ trajectory of the amine group over the course of the rearrangement reaction.

Acknowledgment. This work was supported by NIH Grant DK54514.

Supporting Information Available: $^{14}N-^{15}N$ EPR difference spectra, origins of C1−C2 rotamers, ESEEM simulation parameters, ^1H and ^2H ESEEM spectra, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Banerjee, R. *Chemistry and Biochemistry of B_{12}*; Wiley: New York, 1999.
(2) Banerjee, R.; Ragsdale, S. W. *Annu. Rev. Biochem.* **2003**, *72*, 209−247.
(3) Toraya, T. *Chem. Rev.* **2003**, *103*, 2095−2127.
(4) Semialjac, M.; Schwartz, H. *J. Am. Chem. Soc.* **2002**, *124*, 8974−8983.
(5) Wetmore, S. D.; Smith, D. M.; Bennet, J. T.; Radom, L. *J. Am. Chem. Soc.* **2002**, *124*, 14054−14065.
(6) Bandarian, V.; Reed, G. H. In *Chemistry and Biochemistry of B12*; Banerjee, R., Ed.; Wiley: New York, 1999; pp 811−834.
(7) Babior, B. M.; Moss, T. H.; Orme-Johnson, W. H.; Beinert, H. *J. Biol. Chem.* **1974**, *249*, 4537−4544.
(8) Lobrutto, R.; Bandarian, V.; Magnusson, O. T.; Chen, X.; Schramm, V. L.; Reed, G. H. *Biochemistry* **2001**, *40*, 9−14.
(9) Warncke, K.; Utada, A. S. *J. Am. Chem. Soc.* **2001**, *123*, 8564−8572.
(10) Canfield, J. M.; Warncke, K. *J. Phys. Chem. B* **2002**, *106*, 8831−8841.
(11) Bandarian, V.; Reed, G. H. *Biochemistry* **2002**, *41*, 8580−8588.
(12) Warncke, K.; Schmidt, J. C.; Ke, S.-C. *J. Am. Chem. Soc.* **1999**, *121*, 10522−10528.
(13) Golding, B. T. In *B12*; Dolphin, D., Ed.; Wiley: New York, 1982; Vol. 1, Chapter 15.
(14) (a) Foster, T.; West, P. R. *Can. J. Chem.* **1974**, *52*, 3589−3598. (b) Foster, T.; West, P. R. *Can. J. Chem.* **1974**, *52*, 4009−4017.
(15) Retey, J.; Suckling, C. J.; Arigoni, D.; Babior, B. M. *J. Biol. Chem.* **1974**, *249*, 6359−6360.
(16) Frey, P. A. *Chem. Rev.* **1990**, *90*, 1343−1357.
(17) (a) Tan, S. L.; Kopczynski, M. G.; Bachovin, W. W.; Orme-Johnson, W. H.; Babior, B. M. *J. Biol. Chem.* **1986**, *261*, 3843−3845. (b) Ke, S. C.; Warncke, K. *J. Am. Chem. Soc.* **1999**, *121*, 9922−9927.
(18) Heller, C.; McConnell, H. M. *J. Chem. Phys.* **1960**, *32*, 1535−1539.
(19) Fessenden, R. W.; Schuler, R. H. *J. Chem. Phys.* **1963**, *39*, 2147.

JA031569D