Spectroscopic Evidence for the Participation of an Allylic Analogue of the 5′-Deoxyadenosyl Radical in the Reaction of Lysine 2,3-Aminomutase

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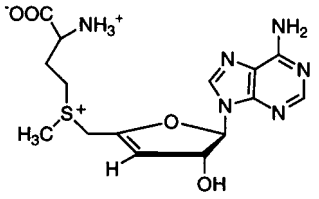
Free radical intermediates participate in the reaction mechanism of lysine 2,3-aminomutase (LAM), and two have been identified as substrate-based free radicals by electron paramagnetic resonance (EPR) spectroscopy.[1−5] An integral component in the proposed mechanism is a 5′-deoxyadenosyl radical derived from *S*-adenosyl-L-methionine (AdoMet). The 5′-deoxyadenosyl radical has never been observed spectroscopically, presumably due to its instability. The cofactor analogue *S*-3′,4′-anhydroadenosyl-L-methionine (3′,4′-anAdoMet) was synthesized by chemical and enzymatic methods (Scheme 1). This compound offers allylic

Scheme 1



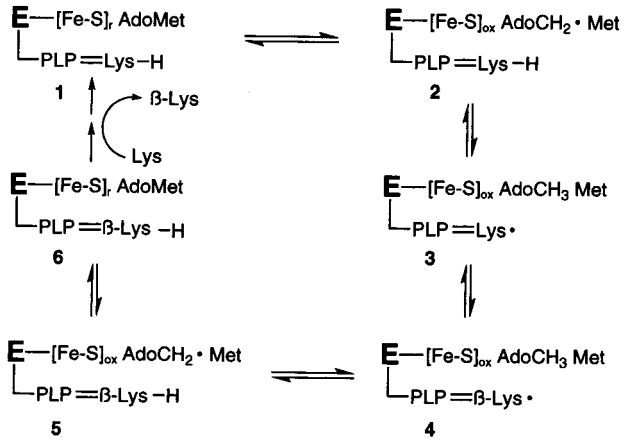
stabilization of a radical formed at the 5′-carbon. 3′,4′-anAdoMet is a cofactor for LAM and displays activity that is about 0.25% of that observed with AdoMet. An organic radical is observed by EPR in samples containing LAM, 3′,4′-anAdoMet, and L-lysine in the steady state of the reaction. Both ^{2}H and ^{13}C labeling experiments provide compelling evidence that the radical is derived from the cofactor analogue.

LAM catalyzes the interconversion of L-α-lysine and L-β-lysine in *Clostridium subterminale* (eq 1). The reaction is the first step

(1)

of a catabolic pathway enabling the bacterium to grow on lysine as its sole source of carbon and nitrogen.[6] This hexameric enzyme contains pyridoxal-5′-phosphate (PLP) and [4Fe-4S] clusters, and it requires the addition of AdoMet for activity. In a process where the [4Fe-4S] clusters donate an electron to AdoMet, the cofactor is postulated to undergo a reductive cleavage to form methionine

Scheme 2



and the 5′-deoxyadenosyl radical **2** (Scheme 2).[7] The unstable primary radical initiates aminomutation by abstracting a hydrogen atom from carbon 3 of lysine to generate the substrate radical **3** and 5′-deoxyadenosine. The substrate radical undergoes rearrangement to the product radical **4** of β-lysine. The 5′-deoxyadenosyl radical is regenerated in the next step **5**, followed by reformation of the cofactor **6** and binding of a new substrate molecule to complete the catalytic cycle. The product radical **4** is observed under steady-state conditions with L-lysine as the substrate and it has been fully characterized by EPR spectroscopy.[1,2,5] The substrate radical **3** has been observed as the dominant radical intermediate with the use of 4-thialysine, an alternative substrate for lysine 2,3-aminomutase.[4] So far, the 5′-deoxyadenosyl radical has not been observed by EPR spectroscopy. The present work describes a preliminary characterization of a stabilized form of the 5′-deoxyadenosyl radical, the allylic species 3′,4′-anhydro-5′-deoxyadenosine-5′-yl, providing the first spectroscopic evidence for the participation of this elusive radical species.

The structure of 3′,4′-anAdoMet is shown in Scheme 1. 3′,4′-Anhydroadenosine was synthesized[8] and enzymatically phosphorylated using adenosine kinase,[9] adenylate kinase, and creatine phosphokinase to generate 3′,4′-anhydroATP,[10] which was subsequently used as a substrate for AdoMet synthetase to produce the final product.[11] LAM was purified from *Clostridium subterminale* SB4 by minor modifications of published procedures.[12]

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(9) The adenosine kinase cDNA in a pET-24b plasmid was the generous gift of Dr. Beverly S. Mitchell. The plasmid was transformed into BL21-(DE3) cells, and the protein expressed and purified as described by: Spychala, J.; Datta, N. S.; Takabayashi, K.; Datta, M.; Fox, I. H.; Gribbin, T.; Mitchell, B. S. *Proc. Natl. Acad. Sci.* **1996**, *93*, 1232−1237.
(10) The reaction was performed in a single process with all three enzymes and catalytic ATP, and it was driven to completion by excess phosphocreatine. The compound was purified by Sephadex A-25 anion exchange chromatography followed by HPLC (Alltech, C18) using 0.2 M KP_i, pH 6, as the mobile phase. Baseline separation between ATP and 3,4-anhydroATP was obtained. The analogue was purified again by HPLC to ensure no ATP contamination. The product was characterized by NMR spectroscopy and mass spectrometry.
(11) A strain, DM22pK8 of AdoMet synthetase was a generous gift of Dr. George D. Markham. The enzyme was expressed and purified as described by: Markham, G. D.; Hafner, E. W.; Tabor, C. W.; Tabor, H. *J. Biol. Chem.* **1980**, *255*, 9082−9092. The AdoMet analogue was synthesized with 3′,4′-anATP and methionine as substrates by modifications of the assay procedure described in the paper. The compound was purified by cation exchange chromatography and characterized by NMR spectroscopy and mass spectrometry.
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Both the standard radiochemical assay and an HPLC method[4] based on derivatization of substrate and product with phenylisothiocyanate were used independently to determine the activity of LAM with 3′,4′-anAdoMet as a cofactor. The compound is a true cofactor for LAM, and with 3′,4′-anAdoMet the enzyme displays a specific activity of 0.10 ± 0.02 IU/mg, which is about 0.25% of that observed with the natural cofactor AdoMet (35−40 IU/mg). These results are in agreement with the formation of a stable 3′,4′-anhydro-5′-deoxyadenosyl radical in the active site of the enzyme. The allylic radical will presumably abstract a hydrogen atom from the substrate (**2** in Scheme 2) at a much slower rate than the more reactive 5′-deoxyadenosyl radical. Inasmuch as hydrogen transfer is rate limiting for the reaction with AdoMet as the cofactor,[13] allylic stabilization should cause a decrease in the overall rate of catalysis. We have also measured a large deuterium isotope effect with 3′,4′-anAdoMet as the cofactor, which supports these conclusions.[14] Allylic substitution of a primary radical can provide up to 11 kcal/mol in stabilization energy,[15] which is more than enough to account for the observed decrease in rate. Stabilization of this magnitude is unlikely in this case for two main reasons: (1) the alignment of the π-orbitals is likely to be distorted due to the constraints imposed by the ring and (2) the enzyme has evolved to bind AdoMet and may interact with a conformer of the analogue that is not the most energetically stable.

EPR spectroscopy was conducted at X-band using a Varian E−3 spectrometer equipped with a standard liquid N_2 immersion dewar to keep the sample temperature at 77 K. The spectrometer was interfaced with an IBM AT computer for data acquisition and analysis. Spin concentrations were determined using a 1 mM $CuSO_4$, 10 mM EDTA standard. The EPR spectra of samples containing LAM, L-lysine, and isotopically labeled forms[16] of the cofactor analogue frozen in cold isopentane in the steady state of the reaction are shown in Figure 1. A new, fairly isotropic EPR signal is observed with 3′,4′-anAdoMet (spectrum A) centered at $g = 2.002$, indicative of a carbon-centered radical. The radical has some resolved hyperfine structure and appears to be homogeneous.[17] Under the experimental conditions, 0.6 radical spins per enzyme hexamer are generated, and the intensity of the signal depends on the presence of the cofactor analogue in a concentration-dependent manner (data not shown). When lysine is omitted, no radical signal is observed (data not shown). This suggests that the reductive cleavage of the cofactor to form the radical and methionine is induced by substrate binding to the enzyme. Spectrum B was acquired with [5′-$^2H_2$]-3′,4′-anAdoMet and lysine-d_8 as a substrate. A change in the hyperfine structure of the signal is consistent with hyperfine coupling from H or D at the 5′-position of the nucleoside moiety. A very similar spectrum was obtained with nonlabeled cofactor analogue and lysine-d_8 (Figure 1C). This can be explained by deuterium enrichment at the 5′-position of the cofactor that occurs after a few turnovers because of the 3′,4′-anhydro-5′-deoxyadenosyl radical acting in accordance with Scheme 2 to abstract a deuterium from carbon 3 of lysine-d_8 as the first step in the mutase reaction. Spectrum D was obtained with [1′,2′,3′,4′,5′-$^{13}C_5$]-anAdoMet. An overall broadening of the spectrum is in accordance with the introduction of a spin 1/2 nuclei coupled to the unpaired π-radical orbital.

LAM belongs to a family of enzymes that use an iron−sulfur cluster and AdoMet to generate a putative 5′-deoxyadenosyl radical, which abstracts a hydrogen atom from carbon. These enzymes include pyruvate formate-lyase activating enzyme,[18] anaerobic ribonucleotide reductase,[19] and biotin synthase.[20] LipA, a protein involved in lipoic acid biosynthesis, has recently been implicated as a candidate in this class of enzymes.[21] AdoMet is a true cofactor of LAM that is required for multiple turnovers, whereas it functions as a cosubstrate in the other reactions.

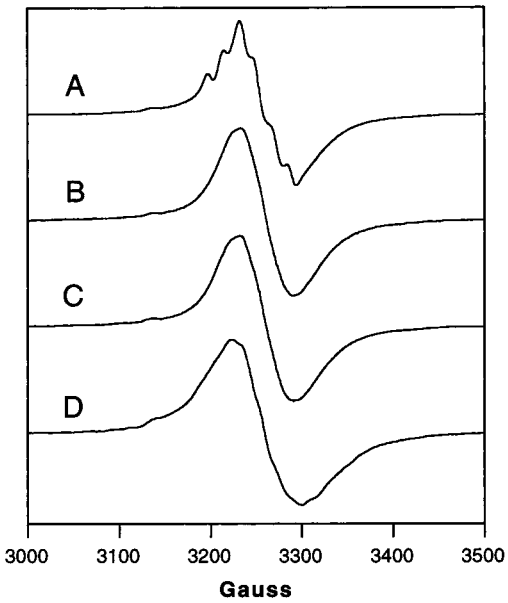

Figure 1. EPR spectra of radical intermediates in the LAM reaction measured at 77 K. All samples contained 50−60 μM hexameric enzyme, 200 mM TrisH_2SO_4, 2 mM dithionite, and the following components: (A) 30 mM L-lysine and 1.0 mM 3′,4′-anAdoMet, (B) 30 mM L-[3,3,4,4,5,5,6,6-$^2H_8$]lysine and 1.0 mM [5′-$^2H_2$]-3′,4′-anAdoMet, (C) 30 mM L-[3,3,4,4,5,5,6,6-$^2H_8$]lysine and 1.0 mM 3′,4′-anAdoMet, and (D) 30 mM L-lysine and 1.0 mM [1′,2′,3′,4′,5′-$^{13}C_5$]-3′,4′-anAdoMet. Instrument settings: scan range, 500 G; microwave frequency, 9.12 GHz; modulation frequency, 100 kHz; modulation amplitude, 4 G; microwave power, 5 mW; time constant 0.3 s; scan time, 4 min.

The 5′-deoxyadenosyl radical has also been proposed as a radical initiator in the coenzyme B_{12}-dependent rearrangements, where it is formed by the homolytic cleavage of the Co−C bond of adenosylcobalamin. This radical, whether derived from AdoMet or adenosylcobalamin, has never been detected by EPR spectroscopy. Based on our data we can unambiguously assign the observed radical to the anhydroribosyl group of the AdoMet analogue, providing the only spectroscopic evidence for a cofactor-derived radical in such a system. Studies in progress are aimed toward deciphering the structure and conformation of this radical in the active site.

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(17) A small feature at $g = 2.08$ (3130 G) is observed in all spectra. This is a component of an axially symmetric signal that appears upon prolonged incubation of the enzyme and is believed to be due to a side reaction off the catalytic pathway.

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