Mechanistic and Spectroscopic Studies of Lysine 2,3-Aminomutase

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Abstract: Lysine 2,3-aminomutase (LAM) catalyzes the interconversion of L-lysine to L-β-lysine using a [4Fe-4S] cluster, S-adenosyl-L-methionine (SAM), and pyridoxal 5'-phosphate (PLP). LAM is a member of the radical-SAM superfamily of proteins which use iron-sulfur clusters and SAM to initiate H atom abstraction reactions. Included in this unusual chemistry is the reductive cleavage of SAM to generate the highly reactive 5'-deoxyadenosyl radical species. This paper will focus on how physical methods, including X-ray absorption spectroscopy (XAS), electron paramagnetic resonance (EPR) and electron-nuclear double resonance (ENDOR) spectroscopies, have provided important information about the structure and mechanism of this remarkable enzyme.

Introduction:

The primary function of iron sulfur clusters is electron transport, as most types of iron sulfur clusters possess at least two readily accessible redox states. One of the most interesting roles of iron sulfur clusters is their role in radical catalysis in the radical SAM superfamily. This protein superfamily is proposed to contain over 600 different enzymes and includes proteins such as lysine 2,3-aminomutase (LAM), biotin synthase (BioB), lipoate synthase (LipA), pyruvate formate lyase activating enzyme (PFL-AE), anaerobic nucleotide reductase activating enzyme (anRNR), and spore photoproduct lyase (SPL). One of the characteristics of the radical SAM superfamily is a conserved cysteine motif, CX\textsubscript{3}CX\textsubscript{2}C, which coordinates an iron-sulfur cluster. With only 3 cysteinal ligands that coordinate the iron-sulfur cluster, these proteins can coordinate either a [3Fe-4S] cluster or a [4Fe-4S] cluster containing one non-cysteinal ligand. The iron sulfur clusters of these enzymes are oxygen sensitive and most are purified under strict anaerobic conditions to maintain activity. These enzymes use S-adenosylmethionine (SAM) to catalyze reactions involving radical intermediates.

LAM was first purified and characterized from *Clostridium subterminale SB4* to study lysine metabolism in *Clostridia*. Lysine 2,3-aminomutase catalyzes the interconversion of L-α-lysine and L-β-lysine. This is the initial step in the catabolism of the amino acid to acetyl-CoA and ammonia, which are usable carbon and nitrogen sources for the bacterium.

\[
\begin{align*}
\text{H}_3\text{N} & \quad \text{H}^+ \\
\text{H} & \quad \text{COOH} \\
\text{H}_3\text{N} & \quad \text{H}^+ \\
\text{H} & \quad \text{COOH}
\end{align*}
\]

LAM is a homohexamer (MW 285 kDa) and functions by a radical mechanism that closely resembles those catalyzed by adenosylcobalamin-dependent enzymes. Similar to reactions activated by AdoCbl, is the participation of the 5'-deoxyadenosyl radical, which initiates free radical formation. However, in LAM, S-adenosylmethionine supplies the 5'-deoxyadenosyl that acts in mediating hydrogen transfer from carbon-3 to carbon-2. This radical initiator occurs from a reversible chemical reaction between SAM and the [4Fe-4S]\textsuperscript{1+} cluster, leading to the cleavage of the C5'-S bond in SAM. Spectroscopic results indicate that this results from reductive cleavage of SAM through electron transfer from the [4Fe-4S]\textsuperscript{1+} cluster, leading to the [4Fe-4S]\textsuperscript{2+} cluster and the 5’dehydroadenosyl radical.

The catalytic mechanism of LAM is shown in Scheme 1. The lysine substrate is bound in an imine linkage with pyridoxal 5'-phosphate (PLP) and isomerization begins by the abstraction of the 3-pro-R hydrogen of lysine by the 5’dehydroadenosyl radical. This produces the substrate radical of α-lysine (radical 1). Proceeding through an aza-cyclopropylcarbinyl radical intermediate (radical 2), the α-lysine-3-yl radical rearranges to the β-lysine-2-yl radical (radical 3). Finally, the 5’dehydroadenosine donates a methyl hydrogen atom back to the β-lysine-2-yl
radical and the pyridoxal-5’-phosphate imine of β-lysine is formed. The regenerated 5’-deoxyadenosyl radical is either ready for the next turnover or recombines with methionine coordinated to the [4Fe-4S] cluster to reform SAM.⁸

![Scheme 1](image)

**Methods**

X-ray absorption spectroscopy (XAS) probes the unoccupied electronic structure of a species. In this work, Selenium K-edge XAS, in combination with the selenium derivative of AdoMet, Se-adenosyl-L-selenomethionine (AdoSeMet) was used to obtain insight into the mechanism of AdoMet cleavage in LAM. Selenium is a good coenzyme for LAM. S-XAS cannot be used as this method “sees” an average of a particular atom and the protein contains many sulfur sites. XAS data was collected at the Stanford Synchrotron Radiation Laboratory (SSRL).⁹

Electron paramagnetic resonance (EPR) requires a paramagnetic species. In this paper, EPR is used to characterize organic radicals. Low temperature EPR spectra were obtained on a Varian spectrometer equipped with an E102 X-band microwave bridge, an Oxford Instruments ESR-900 continuous flow helium cryostat, and an Oxford 3120 temperature controller. EPR measurements at 77K were recorded at X-band on a Varian E-3 spectrometer equipped with a liquid N₂ immersion dewar used to maintain samples at 77K, a temperature at which organic radicals are normally observed in enzymatic systems. Both spectrometers were interfaced with a PC for data acquisition. Scan averaging was employed to improve S/N for subsequent resolution enhancement.¹¹⁻¹³

Electron nuclear double resonance (ENDOR) spectroscopy was used to gain information regarding the interaction of SAM with the [4Fe-4S] cluster. ENDOR spectroscopy is commonly used to resolve smaller hyperfine interactions and can be used to examine specific proton-electron interactions. ENDOR samples were reductively reduced and prepared in an anaerobic chamber. Pulsed ENDOR experiments were carried out at 35 GHz and a temperature of 2 K. Spectra were obtained using SAM labeled with¹⁷O in the carboxylato group, with¹⁵N in the α-amino group, and with either¹³C or²H₃ in the methyl group.¹⁰

**Results**

*X-ray Absorption Spectroscopy*

Se K-edge XAS spectra obtained for selenomethionine (SeMet) and AdoSeMet indicate a difference in the Se oxidation state.⁹,¹⁴ The reduction in the Fourier transform (FT) peak intensity for SeMet indicates a lower coordination number than for that of AdoSeMet. First-shell extended
Figure 2: EPR spectra (77K) of steady-state reaction mixtures with isotopically substituted lysines. Samples contained lysine 2,3-aminomutase (30-35 µM) after 4 h of reductive activation, Tris-HCl at pH 8.0 (50 mM), sodium dithionite (30 mM), SAM (1.2 mM) and the following forms of lysine (200 mM): (a) L-lysine (unlabeled), (b) L-[3,3,4,4,5,5,6,6-2H8]lysine, (c) DL-[2-2H]lysine, and (d) L-[2-13C]lysine. All spectra were accumulated from 20 scans (4 min/scan); the microwave frequency was 9.044 GHz.11

X-ray absorption fine structure (EXAFS) for SeMet have a best fit assuming two carbon atoms at 1.93 Å while first-shell EXAFS for AdoSeMet have a best fit assuming three carbon atoms at 1.94 Å.9

The Se-edge and FT spectra that result from incubating LAM with stoichiometric amounts of AdoSeMet are similar to that of AdoSeMet alone. Incubating LAM with AdoSeMet, dithionite and the substrate analogue, trans-4,5-dehydrolysine, however, results in a Se-edge spectrum similar to SeMet, which indicates cleavage of AdoSeMet to produce SeMet and 5’deoxyadenosine. Observed in this FT spectra, however, is the presence of a new, reproducible peak at ca. 2.7 Å (Figure 2, dotted line). This new peak in the FT at 2.7 Å can be successfully modeled as a first-row transition metal and is interpreted as a selenium-iron interaction with an interatomic distance of 2.67 Å. This distance would be observed as an average of the four Fe atoms in the [4Fe-4S] cluster because XAS “sees” an average coordination environment for all molecules of a given element in the sample. LAM also contains a Zn atom and the Zn K-edge XAS shows the divalent cation site in LAM does not appear to change at any point in catalysis.9

Electron Paramagnetic Resonance

Convincing evidence supporting the mechanism in Scheme 1 has been obtained by electron paramagnetic resonance (EPR) spectroscopy. Using EPR spectroscopy, one of the four participating free radicals in Scheme 1 has been identified as an intermediate, and closely related analogs of two others have also been identified.11-12,15-16 The first free radical to be identified was radical 3 in Scheme 1, the α-radical of the β-lysine side chain. Ballinger et al reported the observation of an EPR signal for an organic radical from a reaction mixture of LAM incubated with SAM and lysine that had been quickly frozen with liquid nitrogen in the steady state of the reaction.16 The intensity of the EPR signal was linearly correlated with enzyme activity and samples prepared with L-[2-2H8]-lysine showed that protons from C3 to C6 of lysine are weakly coupled to the unpaired electron spin.16 Spectra obtained from samples prepared with either [2-2H] lysine or [2-13C] lysine demonstrated that the paramagnetic species is a π-radical with the unpaired electron in a p-orbital on C2 of the lysine skeleton. This was demonstrated through the following: the hyperfine splitting was observed to collapse upon substitution of 2H for 1H at C2 of lysine. Also, introduction of 13C at C2 of lysine results in a new, large splitting of a comparable degree with the central atom hyperfine splittings measured for several π-radicals (Figure 2 c and d).11 It was determined that protons on carbon 4 and 5 were not a significant source of splitting and therefore, the
changes in the hyperfine pattern in spectra of samples with [\( ^{2}\text{H}_\text{a} \)] lysine are solely due to deuterium substitution at the \( \beta \)-carbon (Figure 2 b).

In addition to the \( \alpha \)-proton, the unpaired electron is coupled to the nitrogen of the migratory amino group as well as the \( \beta \)-proton. This became evident upon substitution of \( ^{15}\text{N} \) for \( ^{14}\text{N} \) in the \( \alpha \)-amino group of the substrate. Using either [\( \alpha-^{15}\text{N} \)] lysine or [\( \alpha^{15}\text{N}, 2^{2}\text{H} \)] lysine as a substrate, the resulting EPR spectra exhibit changes in the splitting patterns relative to those in spectra of samples with their respective \( ^{14}\text{N} \) counterparts (Figure 3). Given the location of the unpaired electron on C2 and the presence of the \( \alpha \)-proton, the spin-coupled nitrogen must be attached to the \( \beta \)-carbon. These experimental observations indicate the paramagnetic species is an \( \alpha \)-radical of \( \beta \)-lysine and corresponds to radical 3 in Scheme 1. Using electron spin echo envelope modulation (ESEEM) spectroscopy, it was shown that PLP is covalently linked to the radical. Radical 3 was shown to be kinetically competent as an intermediate.

The unpaired electron on the lysine-related radical intermediate, radical 1 is highly localized on C-3 and is too unstable to exist at a concentration that allows detection by EPR spectroscopy. However, closely related analogs of radical 1 are observed by EPR spectroscopy when functional groups that allow delocalization of the unpaired electron are inserted adjacent to C-3. 4-thia-L-lysine (5-thialysine) is an alternative substrate for LAM as it undergoes normal mutation to 4-thia-L-\( \beta \)-lysine according to eq 2.

The unpaired electron on C-3 is delocalized by the adjacent sulfur atom and is stabilized enough to allow radical 1 to be the only species observed by EPR spectroscopy. The 4-thialysine analog of radical 1 was characterized by the same methods employed for radical 3 in the reaction of lysine. The analog was mixed with SAM and activated LAM and then quickly frozen in liquid nitrogen. The EPR spectrum of radical 1 is shown in Figure 4. The spectrum shown in Figure 4B arises from a sample prepared with 4-thia-DL-[\( ^{2}\text{H}_\text{a} \)] lysine and the hyperfine splitting is significantly narrower than that observed for Figure 4A. This difference would be expected if the unpaired electron resides on C-3 where it would be strongly coupled to the remaining proton at this carbon (in the unlabeled sample). The spectrum observed for Figure 4C generated with
4-thia-L-[3-13C] lysine exhibits a large 13C hyperfine splitting expected for the host carbon nucleus of a π-alkyl radical. The strong hyperfine splitting from 1H and 13C at C-3 indicate the unpaired electron is localized in a p orbital on C-3 of the 4-thialysine skeleton.

Although there has been substantial chemical evidence for the presence of the 5'-deoxyadenosyl radical as a reactive intermediate, it has never been directly observed spectroscopically. The high-energy radical is too unstable to exist at spectroscopically observable concentrations in enzymatic experiments. However, a closely related analog of the 5'-deoxyadenosyl radical, 5'-deoxy-3',4'-anhydroadenosine-5'-yl, can be observed by EPR upon replacement of SAM by S-3',4'-anhydroadenosylmethionine (anSAM). As shown in Scheme 2, the radical resulting from reductive cleavage is stabilized by allylic delocalization.

![Scheme 2](image)

While 77 K is a temperature at which organic radicals are routinely observed in enzymatic systems, the EPR spectrum of the anhydroadenosyl radical at 77 K was irregular. EPR spectra of the anhydroadenosyl radical at 77 K and 4.5 K are shown in Figure 5. The temperature difference has significant effects. At 4.5 K (Figure 5 B) the width of the pattern is more characteristic of a magnetically isolated, organic radical having proton hyperfine splitting. The EPR spectrum of LAM with [13C5-ribosyl] anSAM as the coenzyme, measured at 4.5 K, is shown in Figure 5 C. The substitution of 13C into the anhydroribosyl moiety of the coenzyme analogue significantly changes the EPR signal (Figure 5 B and C). This confirms the location of the unpaired spin on the anhydroribosyl moiety and provides strong support for the suggestion that the 5'-deoxyadenosyl radical is an intermediate in the reaction of LAM.

**Electron Nuclear Double Resonance**

ENDOR spectroscopic studies were employed to show that SAM binds to LAM by chelating the unique iron of the [4Fe-4S] cluster through the carboxylato and amine groups of its L-methionine moiety. The ENDOR spectra of [1+/17O-SAM] show a broad, asymmetric feature which is absent in the unlabeled sample (Figure 6). This signal is assigned to the 17O-carboxylate of SAM. The large coupling at 11.4 MHz is comparable to that of the PLF-AE complex and arises from a carboxylato oxygen coordinated directly

![Figure 5](image)

Figure 5: Effect of temperature and 13C substitution on X-band EPR spectra of the anhydroadenosyl radical. Samples were prepared by mixing LAM with lysine and anSAM at 77K (A) and 4.5 K (B). LAM with lysine and [1',2',3',4',5'-13C] anSAM at 4.5K. Experimental conditions: (A) modulation frequency, 100 kHz; modulation amplitude, 4 G; microwave power 5 mW; spectrometer frequency, 9.13 GHz; time constant, 0.3 s; (B) modulation frequency, 100 kHz; modulation amplitude, 2.5 G; microwave power, 0.02 mW; spectrometer frequency, 9.23 GHz; time constant, 0.3 s; (C) same as in panel B except the modulation amplitude was 3.2 G. All spectra are an average of 4x4 min scans.
to the unique iron of the cluster. The ENDOR spectra of the control (unlabeled [1+/SAM]) shows a $^{14}\text{N}$ signal that disappears in the spectrum of [1+/15$\text{N}$-SAM]. Instead, the 15$\text{N}$ labeled SAM has an ENDOR spectrum with a well-resolved 15$\text{N}$ peak with coupling that requires the amino group of SAM to be coordinated to the unique Fe of the [4Fe-4S]$^{+}$ LAM cluster. The ENDOR data demonstrates that the carboxylato O and amino N of the SAM methionine moiety form a five-membered ring chelate of the unique iron of the LAM [4Fe-4S] cluster, although the details of this chelate appear to be different from that of PFL-AE. Results from $^{13}\text{C}$ ENDOR spectroscopy indicates that the methyl group is closer to Fe in LAM than in PFL-AE, but there is little or no covalent interaction between a negatively charged sulfide of the cluster and the positively charged sulfur of SAM.

**Discussion**

Three of the four proposed radical intermediates of the catalytic cycle of LAM have now been characterized. Upon incubation of LAM with SAM and lysine, the product radical 3 is observed spectroscopically. The signals in the spectra of Figures 2 and 3 appear to come from a single, dominant organic radical. The dominance of this intermediate during the steady state conditions indicates that the α-carbon radical is more stable than the other three radicals shown in Scheme 1. The relative stability of this intermediate can be reasoned by the spin delocalization onto the adjacent carboxylate group since ~80% of the unpaired spin density is located on C2 and spin delocalization onto the carboxylate group likely accounts for the remainder of spin density in the radical. Using 4-thiolsine as a substrate analog, the β-radical (radical 1) can be observed. Interactions of the unpaired electron at C-3 and the nonbonding electrons on sulfur provide adequate stabilization for this substrate radical species to be prevalent under steady-state conditions.

The azacyclopropylcarbinyl radical, radical 2, has not yet been observed by EPR. While delocalization of the unpaired electron onto the pyridinium ring of PLP is a potential stabilizing factor, the strain of the three-membered ring destabilizes it in terms of free energy and thus, prevents it from reaching a detectable concentration.

Several hypothetical mechanisms for the generation of radicals by the reaction of SAM with the [4Fe-4S]$^{+}$ cluster have been suggested. One mechanism with considerable spectroscopic support is based on a direct interaction between the unique iron of the [4Fe-4S] cluster and the sulfonium sulfur of SAM (Scheme 3). The Se-EXAFS results shown above
demonstrate that Se coordinates one of the irons of the Fe-S center with a bond distance of 2.7 Å. These results imply one of the iron sites in the cluster coordinates to a ligand that can be displaced by the sulfur of methionine upon cleavage of the coenzyme. Interestingly, coordination by the Se-methionine moiety of the coenzyme does not occur unless substrate is present. This is also observed by the studies of anSAM where the anhydroadenosyl radical is only observed in the presence of lysine. Together, these results indicate that binding of substrate promotes the reductive cleavage of SAM thus, generating the 5'-deoxyadenosyl radical. The ENDOR data also supports this mechanism.

Reversible cleavage of SAM begins with SAM bound to the unique Fe of the [4Fe-4S] cluster by the carboxylato and amino ligands, with the sulfonium group held close to the cluster. Electron transfer then homolytically cleaves the C5'-S bond of SAM to form the 5'-deoxyadenosyl radical, while the sulfur of methionine becomes the sixth ligand of the unique iron. A six-coordinated octahedral geometry for the unique Fe of a [4Fe-4S] cluster, such as this, is well documented for aconitase. The proposed mechanism allows for reversible cleavage of SAM, as required in the LAM mechanism.

References