S-Adenosylmethionine-Dependent Reduction of Lysine 2,3-Aminomutase and Observation of the Catalytically Functional Iron−Sulfur Centers by Electron Paramagnetic Resonance†

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ABSTRACT: Lysine 2,3-aminomutase catalyzes the interconversion of L-α-lysine and L-β-lysine. The enzyme contains an iron−sulfur cluster with unusual properties, and it requires pyridoxal-5′-phosphate (PLP) and *S*-adenosylmethionine (AdoMet) for activity. The reaction proceeds by a substrate radical rearrangement mechanism, in which the external aldimine formed between PLP and lysine is initially converted into a lysyl-radical intermediate by hydrogen abstraction from C3. The present research concerns the mechanism by which a hydrogen-abstracting species is generated at the active site of lysine 2,3-aminomutase. Earlier tritium tracer experiments have implicated the 5′-deoxyadenosyl moiety of AdoMet in this process. AdoMet is here shown to interact with the iron−sulfur cluster at the active site of Clostridial lysine 2,3-aminomutase. Reduction of the iron−sulfur cluster from its EPR-silent form $[4Fe\text{-}4S]^{2+}$ to the fully reduced form $[4Fe\text{-}4S]^{1+}$ requires the presence of either AdoMet or *S*-adenosylhomocysteine (SAH) and a strong reducing agent such as dithionite or deazariboflavin and light. The reduced forms are provisionally designated **E**−$[4Fe\text{-}4S]^{1+}$/AdoMet and **E**−$[4Fe\text{-}4S]^{1+}$/SAH, and they display similar low-temperature EPR spectra centered at $g_{av} = 1.91$. The reduced form **E**−$[4Fe\text{-}4S]^{1+}$/AdoMet is fully active in the absence of any added reducing agent, whereas the form **E**−$[4Fe\text{-}4S]^{1+}$/SAH is not active. It is postulated that the active form **E**−$[4Fe\text{-}4S]^{1+}$/AdoMet is in equilibrium with a low concentration of a radical-initiating form that contains the 5′-deoxyadenosyl radical. Initiation of the radical rearrangement mechanism is postulated to take place by action of the 5′-deoxyadenosyl radical in abstracting a hydrogen atom from carbon-3 of lysine, which is bound as its external aldiminine with PLP. This process accounts for the results of tritium tracer experiments, it explains the radical rearrangement mechanism, and it rationalizes the roles of AdoMet and the [4Fe-4S] cluster in the reaction.

Lysine 2,3-aminomutase from *Clostridium subterminale SB4* catalyzes reaction 1, the interconversion of L-lysine and L-β-lysine. The enzyme has been characterized as a hexameric protein with an overall molecular mass of 285 kDa and a subunit molecular mass of 47 kDa (*1*, *2*). Although reaction 1 is typical of isomerizations catalyzed by adenosylcobalamin-dependent aminomutases (*3*), the activity of lysine 2,3-aminomutase does not depend upon the presence of adenosylcobalamin. Instead, the coenzymes for reaction 1 are AdoMet[1] and an iron−sulfur cluster, as well as PLP (*1*, *2*, *4*). Adenosylcobalamin-dependent aminomutases also require PLP (*5*).

(1)

The function of adenosylcobalmin in enzymatic isomerization reactions is to initiate radical formation through abstraction of hydrogen atoms from substrates bound at the active sites (*3*). This process is assumed to take place through homolytic scission of the cobalt−carbon bond to form cob(II)alamin and the 5′-deoxyadenosyl radical (Ado-CH_2•). This cleavage is possible at enzymatic sites by virtue of the weakness of the cobalt−carbon bond, about 30 kcal mol^{-1} (*6*, *7*) and the energy that is potentially available through numerous binding interactions between an enzyme and adenosylcobalamin and between the enzyme and its specific substrates. The deoxyadenosyl radical arising from homolytic cobalt−carbon bond cleavage abstracts a hydrogen atom from a substrate to form 5′-deoxyadenosine and a substrate-based radical intermediate, which undergoes isomerization and product formation in subsequent steps.

In the case of lysine 2,3-aminomutase, which does not contain adenosylcobalamin, tritium tracer experiments implicate the deoxyadenosyl moiety of AdoMet in mediating hydrogen transfer. According to the current working hypothesis, the 5′-deoxyadenosyl radical initiates the isomerization by abstracting a hydrogen atom from carbon-3 of the substrate lysine at the active site to form 5′-deoxyadenosine

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[1] Abbreviations: 2,3-aminomutase, lysine 2,3-aminomutase; AdoMet, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; Ado-CH_2•, 5′-deoxyadenosine-5′-yl; PLP, pyridoxal-5′-phosphate; Epps, *N*-[2-hydroxyethyl]-piperazine-*N*′-[3-propanesulfonic acid]; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography.

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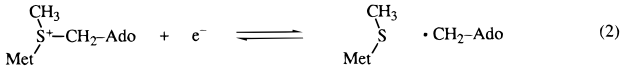
(*8*). The resulting substrate-related radical undergoes an isomerization to a product-related radical, which in turn abstracts a hydrogen from 5′-deoxyadenosine to produce β-lysine and regenerate the 5′-deoxyadenosyl radical. The observation and characterization of the product-related radical by EPR supports this mechanism (*9*). The question of the mechanism by which the 5′-deoxyadenosyl radical might arise through the transient cleavage of AdoMet remains to be answered. A strong bond links the sulfur atom to the 5′-carbon of AdoMet. Because of the strength of this bond, the cleavage of AdoMet to the 5′-deoxyadenosyl radical requires novel chemistry. The reversible cleavage of AdoMet to the deoxyadenosyl radical and methionine is described by reaction 2, which shows that the reaction requires an externally supplied electron. The source of this electron



(2)

within the enzyme−cofactor complex is the subject of the present paper. The AdoMet-dependent reduction of lysine 2,3-aminomutase is reported, and the EPR spectrum of a reduced form of the iron−sulfur cluster is described. The reduced iron−sulfur cluster, which is in an unusual environment, presumably serves as the reductant in reaction 2.

EXPERIMENTAL PROCEDURE

Chemicals. L-Lysine, AdoMet, L-homocysteine thiolactone, and SAH hydrolase were purchased from Sigma. Sodium dithionite was purchased from Aldrich. AdoMet (*p*-toluenesulfonate) was purified by chromatography over a column of CM-cellulose (acetate) equilibrated with 1 mM sodium acetate at pH 5.0 and eluted with 40 mM HCl. Fractions (14−20 mM) were pooled based upon A_{260} (ϵ_{260} = 15 400 M^{-1} cm^{-1}), and aliquots were frozen in liquid N_2 and stored at −70 °C. A portion of the purified AdoMet was diluted to 100 μM in 40 mM HCl, divided into aliquots, frozen in liquid N_2, and stored at −70 °C. [8-^{14}C]Adenosine (specific activity 40−60 Ci/mol) was purchased from American Radiolabel, and [U-^{14}C]lysine was purchased from New England Nuclear. Buffers and other chemicals were purchased in the highest available purity and used as supplied. Buffers were prepared as their potassium or sulfate salts.

Lysine 2,3-Aminomutase. The enzyme was purified from *C. subterminale SB4* inside a Coy anaerobic chamber by a modification of the published procedure (*4*). Samples of enzyme enriched with Co^{2+} at the divalent metal ion binding site were obtained by growing the cells in medium enriched with 35 μM or 0.1 mM $CoCl_2$ and including 10 μM $CoCl_2$ in the purification buffers employed through the Phenyl-Sepharose chromatography step. DTT was replaced with 2-mercaptoethanol in purification buffers when $CoCl_2$ was included. Samples enriched with Zn^{2+} were prepared by the same method with the exclusion of added $CoCl_2$ in cell growth and enzyme purification. The concentration of the purified enzyme ranged from 22 to 37 mg mL^{-1}, and the specific activity was between 20 and 38 units mg^{-1}. All samples of 2,3-aminomutase used for experiments or assays were first subjected to the preliminary reductive incubation described under Preparation of EPR Samples.

Assays. The concentration of purified 2,3-aminomutase was determined spectrophotometrically, using the value ϵ_{280} = 3.6 × 10^5 M^{-1} cm^{-1} (*2*) or by the Bradford method using 2,3-aminomutase as the standard. The enzyme was assayed inside the anaerobic chamber by a modification of the radiochemical method of Chirpich *et al*. (*1*) as amended (*9*). UV−visible spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer. Radiochemical assays were carried out in a Packard Tri-Carb 4640 scintillation spectrometer using 15 mL of Bio-Safe II scintillation fluid (Research Products International) per mL of aqueous solution. Analyses for transition metals were carried out by inductively coupled plasma emission spectroscopy in the Plant and Soil Analysis Laboratory of the University of Wisconsin−Madison. Selected samples were analyzed for iron by the method of Kennedy et al. (*10*). Analyses for inorganic sulfide were carried out as described by Beinert (*11*). HPLC was performed on a system from Waters Chromatography that consisted of 501 and 510 pumps, a 484 tunable absorbance detector, and an automatic gradient controller.

Assays in the Absence of Dithionite. The reaction mixture contained in a final volume of 230 μL: 0.26 M Epps buffer, 2.3 mM Na-dithionite, 1.5 mM AdoMet, and 42 μM enzyme (24 units mg^{-1}). The enzyme was filtered through a column of Sephadex G-25 (1 × 15 cm) equilibrated with 30 mM Epps buffer at pH 8.0, 10 μM PLP, and 0.1 mM L-lysine. A 20-μL aliquot of the desalted protein was diluted to 500 μL with the column buffer, and its activity was determined as described above in the presence as well as the absence of sodium dithionite.

Synthesis of S-[8-^{14}C]Adenosylhomocysteine. [8-^{14}C]SAH was synthesized enzymatically from [8-^{14}C]adenosine and L-homocysteine as described previously (*12*). The reaction mixture included in a volume of 500 μL: 2 mM [8-^{14}C]-adenosine, 50 mM L-homocysteine, 100 mM KP_i (pH 7.2), and 2.5 units of SAH hydrolase. L-Homocysteine was generated from L-homocysteine thiolactone by reaction in 3 M NaOH at room temperature for 20 min and neutralization with HCl. The SAH hydrolase reaction was immediately initiated by addition of L-homocysteine. After 2 h at 37 °C, carrier SAH (0.05 μmol) was added, and the enzyme was precipitated by addition of 500 μL of 0.1 N HCl. After centifugation of the precipitate, SAH was purified by reverse-phase HPLC on a Whatman C18 column (4.6 × 250 mm ZO) with 10% methanol as the solvent at a flow rate of 1 mL min^{-1}. Retention times for SAH and adenosine were 14 and 22 min, respectively. The [8-^{14}C]SAH-fraction was dried by rotary evaporation, redissolved in 0.1 N HCl, rechromatographed under the same conditions, and diluted with SAH.

Assessment of the Integrity of SAH Bound to Reduced Lysine 2,3-Aminomutase. The reaction mixture included the following in a volume of 330 μL: 90 μM [8-^{14}C]SAH (2.7 × 10^6 cpm μmol^{-1}), 140 mM L-lysine, 0.24 M Tris-sulfate at pH 8, 24 μM 2,3-aminomutase, and 2 mM dithionite. After a 10-min incubation at room temperature, 1 mL of 0.1 N HCl was added and the precipitated protein centrifuged. An aliquot of the supernatant fluid (665 μL) was analyzed by HPLC as described above, except that after isocratic elution with 10% methanol for 25 min, a linear gradient from 10% methanol/water to 100% methanol was applied over 15 min.

Fractions (1 mL) were collected and assayed radiochemically.

EPR Measurements. Low-temperature X-band EPR spectra of the iron−sulfur centers were acquired on a Varian E Line spectrometer equipped with a Varian E102 microwave bridge and interfaced with an IBM AT computer. An Oxford Instruments ESR-900 continuous flow helium flow cryostat and Oxford Instruments 3120 temperature controller were used. A Varian gaussmeter and a Hewlett-Packard 5255A frequency converter and 5245L electronic counter were used to measure the magnetic field strength and the frequency, respectively. A Hewlett-Packard 432A power meter was used to calibrate the microwave power. Spectra were acquired as an average of two 16-min or four 4-min scans. Spin concentrations were estimated by double integration of spectra, using a copper perchlorate standard (in 10 mM HCl and 2 M $NaClO_4$) at 10 μW and 13 K as a reference.

X-band EPR spectra at 77 K were obtained as an average of two 4-min scans on a Varian E3 spectrometer interfaced with an IBM AT computer. A standard liquid N_2 immersion dewar (Suprasil Quartz, Wilmad Glass Co.) maintained the sample temperature at 77 K. Spin concentrations were estimated by double integration of EPR spectra, using a Varian strong pitch standard as a reference and *g*-value marker ($g = 2.0028$).

Preparation of EPR Samples. The procedure was a modification of those described earlier (*9*, *13*). Supplies were conditioned to the anaerobic chamber to minimize contamination with air. Purified 2,3-aminomutase contained 30 mM Epps buffer at pH 8.0, 0.1 mM L-lysine, 10 μM PLP, and 1 mM dithiothreitol. In preparation for EPR analysis or activity assays, the enzyme was incubated (4:1) with the reduction buffer, consisting of 0.2 M Tris-sulfate (pH 8.0), 4.8 mM ferric ammonium citrate, 2.3 mM PLP, and 49 mM dihydrolipoate prepared by mixing stock solutions of the components in the order given. After 4 h at 37 °C, aliquots were assayed or prepared for EPR analysis.

In the preparation of EPR samples for reduction with dithionite, the following were added in the order given and to the specified concentrations: lysine to 0.2 M, dithionite to 2 mM, AdoMet or SAH to 1.2−1.8 mM, and 23−45 μM enzyme. Components excluded in controls were replaced with either 0.2 or 1 M Tris-sulfate at pH 8.0. The buffer concentrations in samples varied from 80 to 350 mM. The samples were mixed, transferred to EPR tubes, and frozen by immersion of the tubes in liquid N_2 within 30 s after mixing.

Aliquots of purified 2,3-aminomutase intended for photoreduction were placed in microfuge tubes, frozen with liquid N_2, and stored at −70 °C. For photoreduction, a sample was brought into the anaerobic chamber, thawed quickly, and filtered through a column of G-25 Sephadex (1 × 17 cm) equilibrated with 0.2 M Tris-sulfate at pH 8.0, 10 μM PLP, and 0.1 mM lysine. The enzyme was concentrated using Millipore Ultrafree-MC [30 000 nominal molecular weight limit (NMWL)] vials, and its concentration was determined spectrophotometrically (*2*). One EPR sample was gel filtered, combined with Tris-sulfate (pH 8.0) to 38 μM enzyme and 120 mM buffer, and frozen by immersion of the tube in liquid N_2 within 30 s after mixing.

In the preparation of samples for photoreduction, the following were combined in the given order to the specified concentrations: K-oxalate (pH 5.0) to 17 mM; DTT to 2 mM; and deazariboflavin to 20 μM followed by Tris-sulfate buffer at pH 8.0 (260−350 mM); AdoMet or SAH to 1.7−2.5 mM and 23−41 μM enzyme. Components omitted in controls were replaced with 0.2 or 1 M Tris-sulfate buffer at pH 8.0. Each sample was prepared in the anaerobic chamber in a 1.5 mL microfuge tube, transferred to an EPR tube, capped with a rubber septum, cooled in an ice−water bath, and removed from the chamber for photoreduction with a xenon arc lamp. A water-filled Corex cuvette was placed between the sample and the light as a filter to absorb infrared radiation. The samples for the spectra without added lysine were frozen by immersion of the EPR tube in liquid N_2 immediately after photoreduction. When lysine was added, the photoreduced sample was returned to the anaerobic chamber, lysine was added to a concentration of 0.2 M, and the sample was frozen with liquid N_2 within 30 s after mixing.

Table 1: Transition Metal Ion and Inorganic Sulfide Content of Lysine 2,3-Aminomutase

enzyme preparation	specific activity (IU mg^{-1})	ion content (Mol wt = 285 000) (g atoms mol^{-1})				
		Fe	S^{2-}	Co[a]	Zn[a]	Cu[a]
A[b]	30	12 ± 1		5.3	1.2	0.3
B[b]	35	12 ± 1[c]	11 ± 1	5.0	2.7	0.7
C[d]	36	13 ± 1		3.8	1.0	0.2
D[e]	39	15 ± 2[f]	12 ± 1	0.3	5.3	0.6
E[e]	38	15 ± 2[g]	13 ± 1	0.3	4.7	0.3
F[e]	35	13 ± 1		0.3	5.9	0.4

[a] Experimental uncertainty in these values is estimated to be ±10%. [b] Cell growth medium was supplemented with 0.1 mM $CoCl_2$ and 0.1 mM ferric ammonium citrate. Purification buffers were supplemented with 10 μM $CoCl_2$ through the Phenyl Sepharose chromatography step. [c] Iron analysis by the method of Beinert (*10*) gave a value of 14.0. [d] The cell growth medium was supplemented with 35 μM $CoCl_2$ and 0.1 mM ferric ammonium citrate. Purification buffers were not supplemented with $CoCl_2$. [e] The cell growth medium was supplemented with 0.1 mM ferric ammonium citrate. Purification buffers were not supplemented with $CoCl_2$. [f] Iron analysis by the method of Kennedy et al. (*10*) gave a value of 13.2. [g] Iron analysis by the method of Kennedy et al. (*10*) gave a value of 18.3.

RESULTS

Transition Metal Ions Associated with Lysine 2,3-Aminomutase. Earlier studies showed that 2,3-aminomutase contains iron and acid labile sulfide, as well as a divalent metal ion that can be either cobalt or zinc (*4*). The divalent metal binding site in all preparations contained both metal ions in varying and reciprocal amounts. Samples enriched with cobalt could be prepared by growing *C. subterminale* in a medium supplemented with $CoCl_2$ and adding $CoCl_2$ to the purification buffers. Previous studies indicated that enzymatic activity was maximal with cobalt in the divalent binding site (*4*). The cobalt was found to be present in the form of high-spin cobalt(II) (*14*). Samples of 2,3-aminomutase have been prepared under conditions leading to high cobalt or high zinc content and subjected to analyses for inorganic ions and enzymatic activity, with the results shown in Table 1. The 2,3-aminomutase samples in Table 1 were similar in inorganic ion composition to those reported previously (*4*). However, in contrast to an earlier report, the enzymatic activities of the cobalt- and zinc-enriched samples were similar and did not indicate that catalytic activity depended upon which divalent metal ion was present.

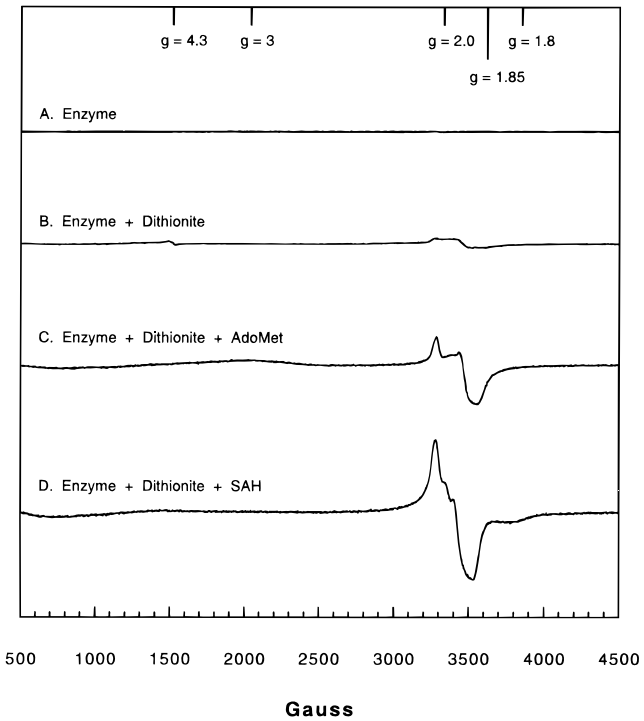


FIGURE 1: EPR spectral evidence for AdoMet- or SAH-dependent reduction of the iron−sulfur cluster in lysine 2,3-aminomutase. Shown are X-band EPR spectra of lysine 2,3-aminomutase acquired at 13 K and normalized to 34 μM 2,3-aminomutase (hexamer): (A) lysine 2,3-aminomutase; (B) lysine 2,3-aminomutase plus dithionite; (C) lysine 2,3-aminomutase plus dithionite and AdoMet; (D) lysine 2,3-aminomutase plus dithionite and SAH. Spectra were recorded at 9.237 GHz, with a modulation amplitude of 4 G at 5 mW power. Gains were normalized to 2.5×10^3. All samples were subjected to the reductive incubation with dihydrolipoate before being further treated with dithionite, dithionite and AdoMet, or dithionite and SAH as described in the Experimental Procedure. Spectra shown are computed averages of two 16-min scans with a time constant of 0.3 s. Spectra of these samples at 77 K in Figure 4 of the Supporting Information show that the sample used for Part C contains a detectable amount of the β-lysyl radical and that used for Part D contains no radical.

Therefore, it must be concluded that the enzymatic activity does not depend on the presence of cobalt in the divalent ion binding site.

AdoMet Dependent Reduction of Lysine 2,3-Aminomutase. A new, heretofore unknown form of the iron−sulfur cluster in 2,3-aminomutase is generated by addition of a strong reducing agent in the presence of AdoMet. The spectrum of this new species is shown in Figure 1C. As shown in Figure 1A, there are no low-temperature EPR signals in the $g = 2$ region of the spectrum of 2,3-aminomutase after reduction with dihydrolipoate to place the iron−sulfur cluster in its EPR-silent form (*12*). Weak EPR signals in the $g = 2$ region appear after addition of dithionite, as shown in Figure 1B. While the nature of this species is not known, it cannot represent an active form of 2,3-aminomutase because the enzyme is not activated by addition of dithionite alone but requires AdoMet as well.

As demonstrated in Figure 1C, addition of both dithionite and AdoMet to the enzyme leads to a new spectrum in the $g = 2$ region. The spectrum near $g = 2$ is more intense and displays much sharper features than that resulting from addition of dithionite alone. This spectrum, centered at a g-value of 1.91 ($g_{av} = 1.91$), is consistent with that of a [4Fe-4S]$^{1+}$ cluster (*15*). A broad feature in the region of $g = 3$ appears in samples containing enzyme, reductant, and AdoMet.

The spectrum in Figure 1D was obtained in a repeat of the experiment in Figure 1C, with substitution of SAH for AdoMet. The most important observation is that the spectrum with SAH is similar to that with AdoMet and is indicative of a [4Fe-4S]$^{1+}$ cluster (*15*). Another significant point is that the spectrum for enzyme/dithionite/SAH is more intense than that for enzyme/dithionite/AdoMet. Double integration under nonsaturating conditions indicates that it corresponds to about 2.2 spins/enzyme molecule (hexamer), which corresponds to the number of [4Fe-4S] centers associated with 2,3-aminomutase (*4*). The spectra differ in other respects, as well. The broad feature centered at $g = 3$ in Figure 1C does not appear when SAH is substituted for AdoMet, but a very broad feature appears in the region of $g = 4-6$ and a small but distinct shoulder at about $g = 1.8$. Saturation studies of the signal in the $g = 2$ region were consistent with one species contributing to the spectrum.

A minor difference between the spectra with AdoMet (Figure 1C) and SAH (Figure 1D) is attributable to a small amount of lysyl radical in the sample containing AdoMet. The buffers used for purification and storage of the enzyme contained 0.1 mM lysine as a stabilizing agent (*16*). The presence of a detectable amount of the lysyl radical in the sample used to record the spectrum in Figure 1C was confirmed in a spectrum recorded at 77 K (Figure 5A Supporting Information), a temperature at which EPR signals from the iron−sulfur centers are too broad to be observed. SAH cannot undergo reduction and initiate the radical chemistry, and the spectrum of the sample used for Figure 1D at 77 K confirmed the absence of a lysyl radical (Figure 5B, Supporting Information).

Effect of Lysine on the EPR Spectra of the Iron−Sulfur Cluster. The EPR spectrum in Figure 2A is that of a sample containing enzyme, AdoMet, dithionite, and lysine at 13 K. The spectrum is similar to that in Figure 1C but with additional features. Most obvious is the off-scale signal at $g = 2.0$ arising from the lysyl radical, which overlies some of the features seen in Figure 1C. The lysyl radical has been identified as an intermediate in the isomerization of lysine to β-lysine (*9*, *13*). That the discontinuity in Figure 2A was due to the lysyl radical was confirmed by its observation at 77 K (Figure 6A, Supporting Information), the temperature at which it had been characterized and at which the spectrum of the iron−sulfur cluster was not observed. In addition, in Figure 2A there are three new weak features at $g = 4.3$, 5.3, and 6.6, which appear only in 2,3-aminomutase samples containing added lysine, AdoMet, and a strong reductant. These are assumed to represent higher spin states of the [4Fe-4S]$^{1+}$ cluster.

Comparison of Figures 1D and 2B shows that added lysine sharpens most of the features in the spectrum of 2,3-aminomutase reduced with dithionite in the presence of SAH, although the shoulder at $g = 1.8$ is weakened. Double integration of the spectrum in Figure 2B indicated about 2.1 spins/enzyme molecule (hexamer), similar to that of Figure 1D. The spectrum of the same sample at 77 K showed no trace of the lysyl radical (Figure 6B, Supporting Information).

EPR Spectrum of Reduced Cobalt-Enriched Lysine 2,3-Aminomutase. As shown in Figure 3, the EPR spectrum in

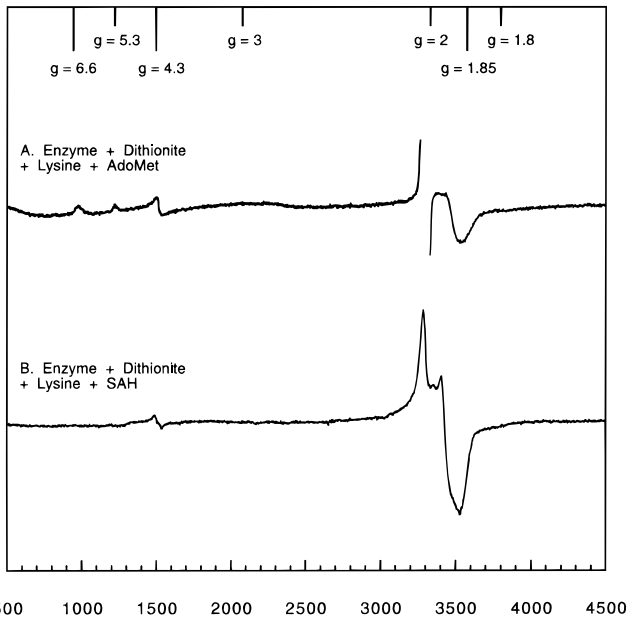


FIGURE 2: Effect of lysine on the low-temperature EPR spectra of 2,3-aminomutase subjected to AdoMet- or SAH-dependent reduction with dithionite. Shown are the X-band EPR spectra at 13 K of lysine 2,3-aminomutase reduced with dithionite in the presence of lysine and either AdoMet or SAH as described in the Experimental Procedure: (A) enzyme plus dithionite, lysine and AdoMet; (B) enzyme plus dithionite, lysine and SAH. Spectra have been normalized for protein concentration (34 μM). Spectra were recorded at 9.237 GHz, with a modulation amplitude of 4 G at 5 mW power. Gains were normalized to 2.5×10^3. Spectra shown are computed averages of two 16-min scans with a time constant of 0.3 s. The break in the spectrum shown in Part A is due to the high intensity (off scale) of the β-lysyl radical spectrum, which is shown on scale at 77 K in Figure 5 (Supporting Information). No lysyl radical appears in Part B, where the spectrum resulting from SAH-dependent reduction is shown.

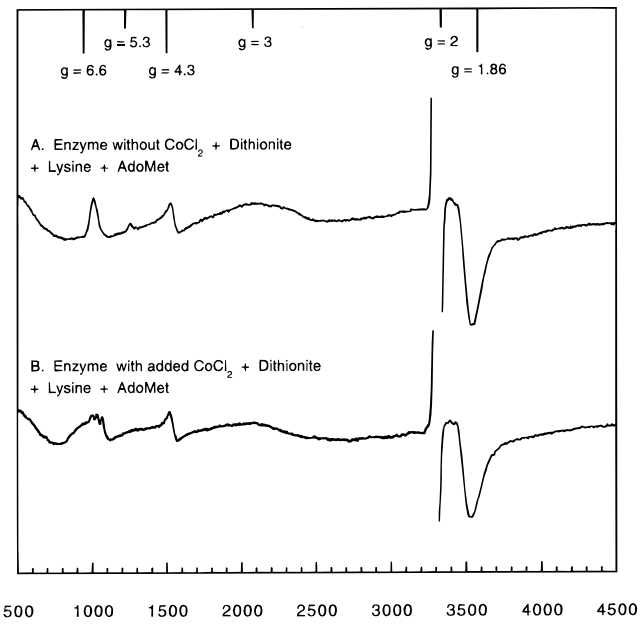


FIGURE 3: Comparison of the low-temperature EPR spectrum of reduced lysine 2,3-aminomutase with that of the reduced cobalt-enriched enzyme. Shown are X-band EPR spectra of lysine 2,3-aminomutase acquired at 4.3 K and normalized to 25 μM enzyme (hexamer): (A) enzyme containing 0.5 Co^{2+} per molecule (hexamer) reduced with dithionite in the presence of AdoMet and lysine as described in Figure 2; (B) enzyme containing 5.0 Co^{2+} per molecule (hexamer) reduced with dithionite in the presence of AdoMet and lysine. Spectra were recorded at 9.24 GHz, with a modulation amplitude of 10 G at 5 mW power. Gains were normalized to 2.5×10^3. The spectra shown are computed average of four 4-min scans with a time constant of 0.3 s.

the $g = 2$ region of the cobalt-enriched enzyme after reduction with dithionite in the presence of AdoMet and lysine is indistinguishable from that of the zinc-containing enzyme. Therefore, it can be concluded that the divalent metal ion binding site is not near enough to the iron−sulfur center to interact magnetically. Furthermore, the typical spectrum for high-spin cobalt(II) in the cobalt-enriched sample, which is absent from the zinc-containing sample, does not reveal any coupling with another paramagnetic center. The EPR spectrum for the lysyl radical also does not indicate any coupling with Co^{2+} in the divalent ion binding site.

EPR Spectra at 13 K of Enzyme Samples with AdoMet or SAH, Reduced by Photoreduction. Figure 4 depicts spectra acquired from samples reduced by photoreduction with deazariboflavin. Samples of 2,3-aminomutase treated with this reducing system display EPR spectra similar to those treated with dithionite. In all photoreduced samples, a spectral envelope appears in the $g = 2$ region, partially attributed to reduced iron−sulfur centers, as well as in the position of $g = 4.3$. Although light-reduced deazariboflavin is the stronger reductant (*17*, *18*), it does not seem to reduce the iron−sulfur centers as effectively as dithionite. Neither increasing the amount of deazariboflavin nor increasing the time of light exposure leads to greater spectral intensity.

The spectrum in Figure 4A is that of a photoreduced sample containing enzyme, light-reduced deazariboflavin and SAH. The features of this spectrum are similar to those of a parallel sample reduced by dithionite (Figure 1D). The two spectra differ by the presence of a weak signal at $g = 4.3$ in the deazariboflavin-reduced sample. Double integration of the spectrum in Figure 4A in the $g = 2$ region indicated about 1.1 spins/enzyme molecule.

The EPR spectrum of 2,3-aminomutase photoreduced in the presence of AdoMet is shown in Figure 4B. A cursory examination of Figure 4B compared with Figure 1C, in which dithionite was the reductant, reveals two differences. First, the overall spectral intensity of the deazariboflavin-reduced sample (Figure 4B) appears to be less than that of the dithionite-reduced sample (Figure 1C). The most obvious difference in the features in the $g = 2$ region can be attributed to the lysyl radical spectrum in the deazariboflavin-reduced sample (Figure 7B, Supporting Information), which is weaker in the dithionite-reduced sample (Figure 5A, Supplementrary Material); the relatively stronger lysine radical spectrum influences the spectral line shape of the deazariboflavin-reduced sample. Despite these distinctions, each spectrum contains the same major features at 13 K.

Addition of lysine to a sample of enzyme containing AdoMet and deazariboflavin after photoreduction led to the EPR spectrum shown in Figure 4C. The relative intensities of most features are similar to those in Figure 4B with one exception. The sharp line at $g = 2$ is enhanced by the addition of lysine, and its prominence is attributed to the

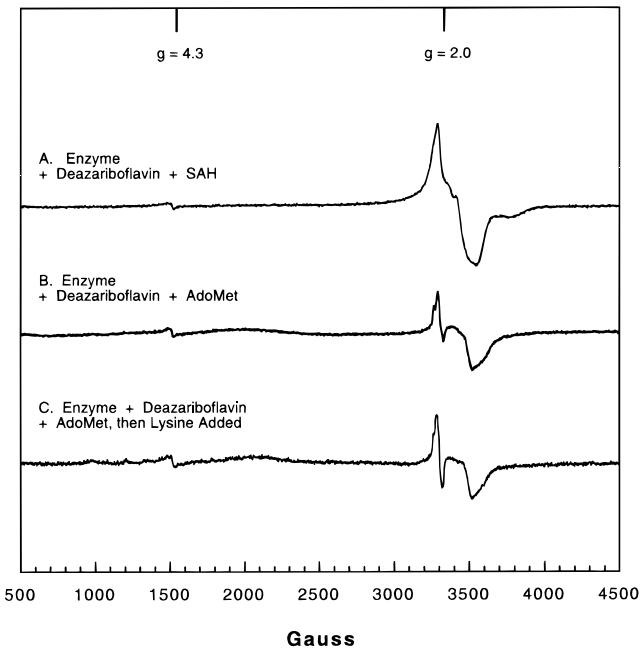


FIGURE 4: EPR spectral evidence for AdoMet- or SAH-dependent photoreduction of the iron−sulfur cluster in lysine 2,3-aminomutase. Lysine 2,3-aminomutase was photoreduced with deazariboflavin in the presence of AdoMet or SAH, or in the presence of AdoMet with lysine added after photoreduction as described in the Experimental Procedure. Spectra have been normalized for protein concentration (23 μM). The samples are as follows: (A) enzyme photoreduced in the presence of deazariboflavin and SAH; (B) enzyme photoreduced in the presence of deazariboflavin and AdoMet; (C) enzyme photoreduced in the presence of deazariboflavin and AdoMet, with lysine added after photoreduction. Spectra were recorded at 9.24 GHz, with a modulation amplitude of 4 G at 5 mW power. Gains were normalized to 3.2×10^3. Spectra shown are computed averages of two 16-min scans with a time constant of 0.3 s. The spectra in Parts B and C include the β-lysyl radical spectrum, as shown by the spectra at 77 K in Figure 6 (Supporting Information).

increased contribution of the lysyl radical spectrum, as shown by the 77 K EPR spectrum (Figure 7C, Supporting Information). Comparison of the spectrum in Figure 4C (photoreduction) with that in Figure 2A (dithionite) shows that the photoreduced sample displays the features of the dithionite-reduced sample but with less intensity. The lower intensity and the low intensity of the signal of the lysyl radical relative to analogous samples reduced with dithionite is attributed to experimental differences. Dithionite-reduced samples contained excess reductant and remained inside the anaerobic chamber until addition of lysine, whereas photoreduced samples received one cycle of reduction outside the anaerobic chamber and were then transferred into the chamber before being opened for the addition of lysine. The reduced cofactor $[4Fe\text{-}4S]^{1+}$/AdoMet underwent significant decomposition in the intervening time. Release of methionine from the active complex has been described in earlier work (*16*).

Role of Dithionite in the Action of Lysine 2,3-Aminomutase. In earlier experiments, dithionite has been required for enzymatic activity or to generate the lysyl radical and the $[4Fe\text{-}4S]^{1+}$ species. In experiments carried out in the anaerobic chamber, the dihydrolipoate-treated enzyme was gel filtered and assayed in the presence or absence of dithionite. The activity in the presence of dithionite was 9000 times that in its absence, which was our limit of detection. Control experiments showed that inactivity in the absence of dithionite was not due to inactivation of the enzyme by adventitious oxygen. In addition, the EPR spectra at 13 K showed no evidence of significant oxygen-mediated destruction of the iron−sulfur cluster in either sample, as indicated by the absence of signals at $g = 4.3$ region, and both samples remained EPR silent. These results suggest that dithionite functions in some capacity in addition to that of an oxygen scavenger. Dithionite is a powerful reductant ($E^{\circ\prime} = -0.660$ V) (*19*), and its requirement for activity means that it must play some essential role.

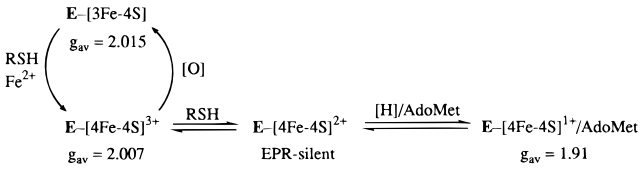
To determine whether dithionite participates directly in catalysis, the enzyme was treated with dithionite and AdoMet and immediately gel filtered to remove excess AdoMet and reductant. A portion of the protein fraction was diluted into the column buffer for assays in the presence and absence of dithionite. EPR analysis of the remainder showed the spectrum of the $[4Fe\text{-}4S]^{1+}$ cluster at 13 K. Most important, however, the activity of the enzyme without added dithionite (18 units mg protein^{-1}) was only slightly less than that in the presence of dithionite (20 units mg protein^{-1}). To determine whether the activity was due to dithionite contamination from the gel filtration column, a similar sample was reconcentrated using a Centricon 30 ultrafiltration device, rechromatographed on another column of Sephadex G-25, and assayed in the absence of dithionite. Full activity (21 units mg^{-1}) was observed. This experiment proved that the dithionite requirement for activity was abolished by the presence of AdoMet during the reduction. The simplest explanation for the dithionite requirement is that it participates in the AdoMet-dependent reduction of the iron−sulfur cluster to $[4Fe\text{-}4S]^{1+}$/AdoMet; once the cluster is in this form, the enzyme is active.

The activity of the $[4Fe\text{-}4S]^{1+}$/AdoMet form of the enzyme under anaerobic conditions at ambient temperature was measured in the absence of dithionite at 4-h intervals over 22 h. No more than 30% of the initial activity was lost. This result shows that the $[4Fe\text{-}4S]^{1+}$/AdoMet form of the enzyme is a remarkably stable species.

Integrity of SAH upon Reduction of the Iron−Sulfur Complex to [4Fe-4S]$^{1+}$. To examine whether the AdoMet- or SAH-dependent reduction of the iron−sulfur cluster to $[4Fe\text{-}4S]^{1+}$ was accompanied by cleavage of the adenosyl cofactor, the interaction of 2,3-aminomutase with SAH was examined further. The enzyme was treated with dithionite and SAH and gel filtered as described above for the AdoMet-dependent reduction. The filtered protein displayed the EPR spectrum typical of SAH-dependent reduction (Figure 1D). When assayed in the presence of saturating AdoMet, no activity was detected. Control samples with SAH omitted displayed activity when assayed with addition of AdoMet, suggesting that SAH remained bound to the enzyme in the test experiment. Activity assays with added AdoMet at intervals of 2 min over an 8-min time span showed no increase in activity, indicating that SAH-dissociation was slow at 37 °C.

To determine whether the adenosyl cofactor was cleaved upon reduction of the iron−sulfur complex, [*adenosyl*-8-^{14}C]-SAH was used in the reduction with dithionite, and the reduced enzyme complex was isolated by gel filtration. Radiochemical analysis of protein-containing fractions indicated the presence of 0.7 SAH/molecule of enzyme (hexamer). EPR analysis for $[4Fe\text{-}4S]^{1+}$ of an identical

Scheme 1



sample using unlabeled SAH gave approximately 0.45 spin/molecule (hexamer). After acid denaturation of the reduced enzyme complex prepared with [*adenosyl*-8-^{14}C]SAH, HPLC and radiochemical analysis of the soluble ^{14}C-labeled material showed that all of the radioactivity migrated with SAH. Therefore, SAH-dependent reduction of the iron−sulfur center in 2,3-aminomutase to [4Fe-4S]$^{1+}$ was not accompanied by a chemical cleavage of SAH.

DISCUSSION

The rearrangement of lysine into β-lysine by 2,3-aminomutase proceeds by a radical mechanism through lysyl radical intermediates. The lysine- and the β-lysine-related radicals have been observed by EPR and ESEEM spectroscopy (*9*, *13*, *20*, *21*), and the β-lysine-related radical has been found to be kinetically competent (*22*). The lysyl radicals must arise by hydrogen abstraction from external aldimines of PLP with lysine or β-lysine. The 5′-deoxyadenosyl moiety of AdoMet mediates hydrogen transfer and, in the form of the 5′-deoxyadenosyl radical (AdoCH$_2$•), is postulated to be the hydrogen-abstracting initiator of the radical rearrangement (*8*, *16*). The question of the chemistry by which AdoMet can be reversibly cleaved to AdoCH$_2$• (eq 2) remains to be established.

Paramagnetic States of the Iron−Sulfur Center. The iron−sulfur center in 2,3-aminomutase has been observed by EPR spectroscopy in three states. In earlier work of Petrovich et al. (*14*), the form designated as [4Fe-4S]$^{3+}$ in Scheme 1 was characterized. Controlled oxidation resulted in the form [3Fe-4S], which was characterized by EPR. Treatment of the enzyme containing the [4Fe-4S]$^{3+}$ cluster with dihydrolipoate converted the iron−sulfur center into an EPR-silent state.

The evidence indicating that the iron−sulfur cluster is of the [4Fe-4S]-type consists of the following: (a) the iron−sulfur center can be transformed into a [3Fe-4S] cluster by controlled oxidation and then reconstituted by addition of a sulfhydryl agent and Fe^{2+} (*14*); (b) the EPR spectrum of the form designated [4Fe-4S]$^{3+}$ is compatible with a four-iron and not a two-iron cluster (*14*); (c) In work described here, a fully reduced form can be produced by AdoMet-dependent reduction, and its EPR spectrum is characteristic of [4Fe-4S]$^{1+}$ (*15*). Therefore, as a working hypothesis, the accessible oxidation states for the iron−sulfur cluster are designated in Scheme 1 as the three known oxidation states for [4Fe-4S] clusters. Controlled oxidation converts the [4Fe-4S]$^{3+}$ cluster into the [3Fe-4S] cluster shown in Scheme 1.

Evidence in this paper suggests that the catalytically functional species of the iron−sulfur center is the one produced by a strong reducing agent in the presence of AdoMet, designated as [4Fe-4S]$^{1+}$/AdoMet in Scheme 1. This form displays catalytic activity even when separated from dithionite. The forms [4Fe-4S]$^{2+}$ and [4Fe-4S]$^{3+}$ in Scheme 1 are inactive.

An analogue of the active form, [4Fe-4S]$^{1+}$/AdoMet, is produced by the substitution of SAH for AdoMet in the reduction by dithionite. The [4Fe-4S]$^{1+}$/SAH analogue is not catalytically active, nor can it produce the lysyl radical, which has been shown to be an intermediate in the isomerization of lysine. Nevertheless, the EPR spectrum of [4Fe-4S]$^{1+}$/SAH is similar to that of the active species [4Fe-4S]$^{1+}$/AdoMet, and the formation of the two complexes takes place under the same conditions. The fact that SAH is not cleaved in [4Fe-4S]$^{1+}$/SAH suggests that the cleavage of AdoMet is not required to reduce the iron−sulfur center within the active form [4Fe-4S]$^{1+}$/AdoMet.

Although cleavage of the adenosyl cofactor is not required, either AdoMet or SAH is required for the reduction of the iron−sulfur center to [4Fe-4S]$^{1+}$. The simplest explanation for these observations is that the adenosyl cofactor facilitates the reduction of the EPR-silent form [4Fe-4S]$^{2+}$, presumably through binding interactions at the AdoMet site. Reduction of [4Fe-4S]$^{2+}$ might be facilitated by an electrostatic interaction of the sulfonium group of AdoMet with the iron−sulfur center; however, because the reduction is also promoted by SAH, which lacks the sulfonium ion, an electrostatic interaction cannot be a major driving force for reduction. Binding of the adenosyl and methionyl portions of AdoMet, both of which are present in SAH, appears to be important in potentiating reduction of the EPR-silent form by dithionite. 5′-Deoxyadenosine and methionine, either alone or together, do not promote the reduction.

Participation of the Iron−Sulfur Cluster in Radical Formation. The active form of 2,3-aminomutase, [4Fe-4S]$^{1+}$/AdoMet in Scheme 1, presumably allows the transient formation of the 5′-deoxyadenosyl radical. This proposition is supported by its catalytic activity and its ability to generate the intermediate β-lysyl radical. This form of the enzyme could initiate radical formation by the reversible transformation in reaction 3. The deoxyadenosyl radical on the right

$$\text{E}-[\text{4Fe-4S}]^{1+}/\text{AdoMet} \rightleftharpoons \text{E-}[\text{4Fe-4S}]^{2+}/\text{Met/Ado-CH}_2\bullet \quad (3)$$

side could initiate the postulated isomerization mechanism by abstracting a hydrogen atom from the external aldimine of lysine or β-lysine (*8*, *9*, *13*, *20*−*22*). The equilibrium for eq 3 must lie far to the left because of the instability of Ado-CH$_2$•, which has never been observed in EPR experiments.

The process of eq 3 presumably also takes place in the enzymatic activation of *E. coli* PFL and ARR. The active forms of these two enzymes contain glycyl radicals within their polypeptide chains (*23*, *24*). ARR and PFL are activated through the transformation of glycyl redidues into glycyl radicals by activating enzymes that require AdoMet and iron−sulfur clusters. Glycyl radical formation is accompanied by the transformation of AdoMet into methionine and 5′-deoxyadenosine concomitant with the abstraction of an α-hydrogen atom from a glycyl residue. The process of reaction 3 presumably allows for the transient formation of the 5′-deoxyadenosyl radical, which abstracts hydrogen from the glycyl residues. Iron−sulfur species analogous to [4Fe-4S]$^{1+}$ in Scheme 1 have been observed in these systems (*25*, *26*).

Lysine 2,3-Aminomutase as a Unique Metalloprotein. To our knowledge, lysine 2,3-aminomutase is the only protein

containing [4Fe-4S] clusters in which the iron−sulfur center has been shown to exist in all three known oxidation states. Clusters of the [4Fe-4S] type in other proteins are generally described as either $[4Fe\text{-}4S]^{3+}/[4Fe\text{-}4S]^{2+}$, characteristic of high potential iron−sulfur proteins, or as the ferredoxin-type clusters $[4Fe\text{-}4S]^{2+}/[4Fe\text{-}4S]^{1+}$. In the case of 2,3-aminomutase, the observation of the most highly reduced form requires the presence of AdoMet or SAH. The $[4Fe\text{-}4S]^{3+}$ cluster and the [3Fe-4S] cluster derived from it by controlled oxidation are unlikely to exist in the cell or to participate in the action of 2,3-aminomutase. Presence of the $[4Fe\text{-}4S]^{3+}$ cluster in vitro in the purified enzyme most likely is brought about by adventitious oxidation of the $[4Fe\text{-}4S]^{2+}$ cluster. Inasmuch as the active form of the cluster is $[4Fe\text{-}4S]^{1+}$/AdoMet, and this is formed by AdoMet-dependent reduction of $[4Fe\text{-}4S]^{2+}$, the latter two forms are likely to be biologically significant.

SUPPORTING INFORMATION AVAILABLE

Three figures (Figures 5−7) of X-band EPR spectra of lysine 2,3-aminomutase (5 pages). Ordering information is given on any current masthead page.

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