Characterization of Iron–Sulfur Clusters in Lysine 2,3-Aminomutase by Electron Paramagnetic Resonance Spectroscopy†

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Received June 16, 1992; Revised Manuscript Received August 28, 1992

ABSTRACT: Lysine 2,3-aminomutase from *Clostridia* catalyzes the interconversion of L-α-lysine with L-β-lysine. The purified enzyme contains iron–sulfur ([Fe-S]) clusters, pyridoxal phosphate, and Co(II) [Petrovich, R. M., Ruzicka, F. J., Reed, G. H., & Frey, P. A. (1991) *J. Biol. Chem. 266*, 7656–7660]. Enzymatic activity depends upon the presence and integrity of these cofactors. In addition, the enzyme is activated by *S*-adenosylmethionine, which participates in the transfer of a substrate hydrogen atom between carbon-3 of lysine and carbon-2 of β-lysine [Moss, M., & Frey, P. A. (1987) *J. Biol. Chem. 262*, 14859–14862]. This paper describes the electron paramagnetic resonance (EPR) properties of the [Fe-S] clusters. Purified samples of the enzyme also contain low and variable levels of a stable radical. The radical spectrum is centered at $g = 2.006$ and is subject to inhomogeneous broadening at 10 K, with a $p_{1/2}$ value of 550 ± 100 μW. The low-temperature EPR spectrum of the [Fe-S] cluster is centered at $g = 2.007$ and undergoes power saturation at 10 K in a homogeneous manner, with a $p_{1/2}$ of 15 ± 2 mW. The signals are consistent with the formulation [4Fe-4S] and are adequately simulated by a rhombic spectrum, in which $g_{xx} = 2.027$, $g_{yy} = 2.007$, and $g_{zz} = 1.99$. Treatment of the enzyme with reducing agents converts the cluster into an EPR-silent form. Oxidation of the purified enzyme by air or ferricyanide converts the [Fe-S] complex into a species with an EPR spectrum that is consistent with the formulation [3Fe-4S]. The signal of the [3Fe-4S] cluster undergoes power saturation at 10 K in a homogeneous fashion, in which $p_{1/2} = 10$ ± 3 mW. The EPR spectrum of the [3Fe-4S] cluster is simulated by a rhombic spectrum, in which $g_{xx} = 2.032$, $g_{yy} = 2.015$, and $g_{zz} = 2.0125$. Double integration of the [3Fe-4S] spectrum indicates the presence of 2.5–3 spins per hexameric unit of enzyme. Reductive activation of the enzyme leads to the disappearance of the EPR signal of the [4Fe-4S] cluster, at a rate that is significantly faster than the activation process, and to the quenching of the radical signal at a rate that is comparable to the rate of activation. The reduction potential of the [4Fe-4S] cluster is more positive than −370 mV.

Lysine 2,3-aminomutase catalyzes the first step in the metabolism of lysine in *Clostridia*, the conversion of L-α-lysine into L-β-lysine (Chirpich et al., 1970; Stadtman, 1973). The enzyme purified from *Clostridium* SB_4 is a hexamer of apparently identical subunits with a molecular weight of 285 000, and it contains iron, inorganic sulfide, cobalt, and PLP[1] (Chirpich et al., 1970; Petrovich et al., 1991; Song & Frey, 1991). In the course of purification, the enzyme loses activity and must be reactivated by incubation for 5 h at 37 °C in the presence of Fe(II), PLP, and a thiol-reducing agent such as glutathione or dihydrolipoic acid, followed by the addition of *S*-adenosylmethionine, all under strictly anaerobic conditions (Chirpich et al., 1970; Moss & Frey, 1987). Although the interconversion of lysine and β-lysine is chemically analogous to the rearrangements catalyzed by adenosylcobalamin-dependent aminomutases, such as β-lysine mutase and ornithine mutase (Baker & Stadtman, 1982), lysine 2,3-aminomutase does not contain a B_{12} cofactor (Petrovich et al., 1991).

The adenosyl moiety of *S*-adenosylmethionine takes part in mediating the transfer of a hydrogen atom from carbon-3 of lysine to carbon-2 of β-lysine (Moss & Frey, 1987; Baraniak et al., 1989). Chemical studies indicate that activation of lysine 2,3-aminomutase by *S*-adenosylmethionine includes the reaction of *S*-adenosylmethionine with a reduced metal cofactor in the presence of lysine to produce an adenosylcofactor and methionine according to Scheme I (Moss & Frey, 1990). In a hypothesis recently advanced to explain the activation of this enzyme (Petrovich et al., 1991), the adenosylcofactor is postulated to undergo a reversible cleavage to the 5′-deoxyadenosyl radical, and this radical initiates the rearrangement by abstracting the 3-*pro-R* hydrogen atom of the lysine–PLP aldimine to form 5′-deoxyadenosine and the β-radical of lysine–PLP aldimine, which undergoes a rearrangement to the α-radical of β-lysine–PLP aldimine. The α-radical abstracts a hydrogen atom from 5′-deoxyadenosine to produce β-lysine–PLP aldimine and regenerate the 5′-deoxyadenosyl radical, which can reform the adenosylcofactor. The existence of a lysine-based radical intermediate, the generation of 5′-deoxyadenosine and methionine, and participation by the adenosyl-5′-methylene group in *S*-adenosylmethionine in the hydrogen transfer mechanism have all been demonstrated (Ballinger et al., 1992; Moss & Frey, 1987; 1990; Baraniak et al., 1989; Kilgore & Aberhart, 1991).

The mechanism of the lysine 2,3-aminomutase reaction may be analogous to the adenosylcobalamin-dependent aminomutase reactions, in which adenosylcobalamin is thought to undergo reversible cleavage to the 5′-deoxyadenosyl radical and B_{12r}[Co(II)] (Abeles & Dolphin, 1976; Golding, 1982; Frey, 1990). According to this hypothesis, the metal cofactors

† Supported by Grant DK28607 from the National Institute of Diabetes and Digestive and Kidney Diseases (to P.A.F.) and Grant GM35752 (to G.H.R.) from the National Institute of General Medical Sciences.

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; B_{12r}, vitamin B_{12} with cobalt in the +2 oxidation state; EPR, electron paramagnetic resonance; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

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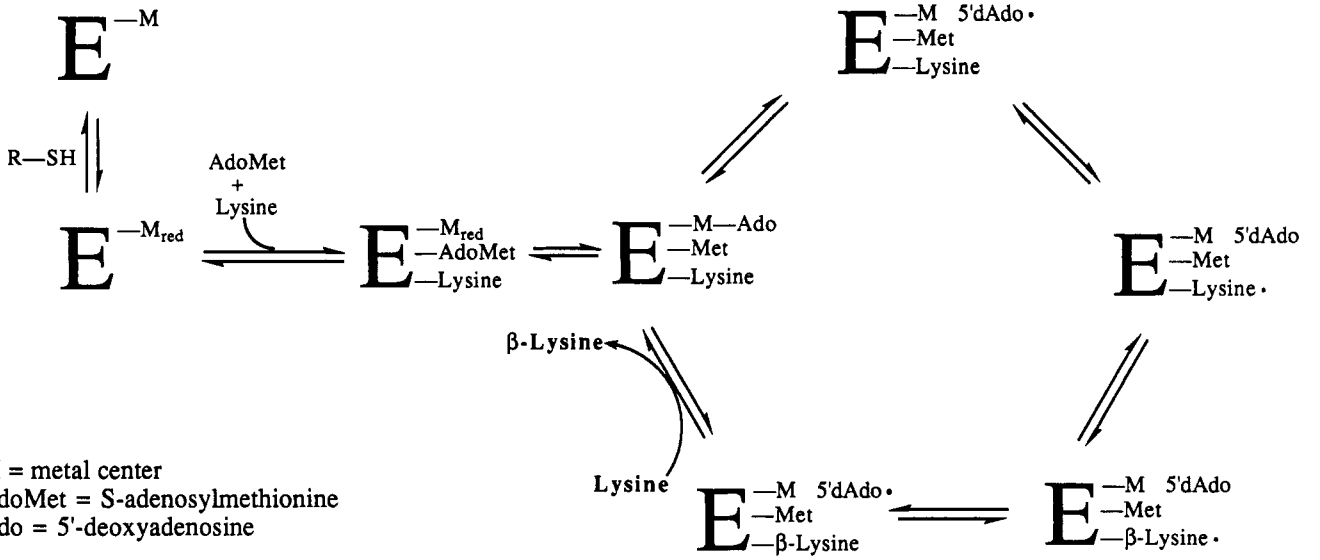
Scheme I



M = metal center
AdoMet = S-adenosylmethionine
Ado = 5'-deoxyadenosine

of lysine 2,3-aminomutase react with *S*-adenosylmethionine to form a factor that has at least one property in common with adenosylcobalamin, the reversible generation of the 5'-deoxyadenosyl radical. However, the metallic centers in the lysine 2,3-aminomutase have not been characterized.

The iron centers in lysine 2,3-aminomutase have been postulated to be [Fe-S] clusters on the basis on the visible spectrum of the enzyme and the presence of stoichiometrically comparable amounts of protein-bound iron and sulfide (Petrovich et al., 1991). In this paper, we describe the EPR properties of the [FeS] clusters in lysine 2,3-aminomutase.

EXPERIMENTAL PROCEDURES

Growth of Clostridium SB4. Cells were grown as described by Chirpich et al. (1970), except that the media were supplemented with ferric ammonium citrate and cobalt chloride, both at 100 μM. The cell cultures were harvested at turbidities corresponding to A_{660} values of 0.5–0.7.

Purification of Lysine 2,3-Aminomutase. The enzyme was purified following the procedure of Moss and Frey, (1990), as amended by Petrovich et al., (1991) using standard isolation buffer (30 mM Tris-HCl at pH 8.0, 1 mM dithiothreitol, 0.1 mM lysine, and 0.01 mM PLP) and cobalt-supplemented isolation buffer (30 mM Tris-HCl at pH 8.0, 1 mM mercaptoethanol, 10–100 μM $CoCl_2$, 0.1 mM lysine, and 0.01 mM PLP). Mercaptoethanol replaced dithiothreitol in the cobalt-supplemented buffer to prevent the precipitation of cobalt–dithiothreitol. The purification was carried out in a Coy anaerobic chamber through the final step of ion-exchange chromatography over a 50-mL fast flow Q-Sepharose column. Cobalt-supplemented isolation buffer was used through the phenyl-Sepharose chromatography step, and the standard isolation buffer was used in the final Q-Sepharose step.

Activation and Assay of Lysine 2,3-Aminomutase. The activity of dilute lysine 2,3-aminomutase (1 μM to 1.6 μM in the reductive activation solution) was measured by a modification of the procedure of Chirpich et al., (1970), in which 500 μL Eppendorf microfuge tubes replaced the 50-μL flame-sealed capillary tubes, and the entire procedure was carried out inside a Coy anaerobic chamber. This procedure eliminated the requirement for sodium dithionite in the preliminary reductive activation, but sodium dithionite was still required when lysine was being converted to β-lysine in the presence of *S*-adenosylmethionine and activated enzyme. A 5-h preliminary incubation in the presence of 15 mM dihydrolipoic acid, 1 mM Fe(II), 0.4 mM PLP, and 42 mM Tris-HCl at pH 8.0 was required to achieve maximal activity in the presence of 8.3 μM *S*-adenosylmethionine (Moss & Frey, 1987).

The activity of concentrated lysine 2,3-aminomutase (37 μM in the reductive activation solution) was measured by a modification of the procedure of Ballinger et al. (1992). At this concentration of enzyme there was a requirement for Fe(II) (1 mM Fe(II) yielded the maximal activity) in the preliminary reductive activation solution. As in activation of the enzyme at lower concentrations. there was no requirement for sodium dithionite in the preliminary reductive activation. However, sodium dithionite was still required to maintain enzymatic activity in the course of the conversion of lysine into β-lysine in the presence of *S*-adenosylmethionine.

Metal Analysis. Metal analyses were carried out by inductively coupled plasma emission spectroscopy in the Plant and Soil Analysis Laboratory of the University of Wisconsin–Madison.

EPR Spectroscopy. Low-temperature EPR spectra were obtained with a Varian E Line spectrometer equipped with a Varian E102 microwave bridge. Precise measurements of the microwave frequency and the magnetic field strength were obtained using a Hewlett Packard 5255A frequency converter (3–12.4-GHz range)/5245L electronic counter and a Varian gaussmeter, respectively. The microwave power was calibrated using a Hewlett Packard 432A power meter. The temperature was regulated using an Oxford ESR-900 continuous flow cryostat and monitored with an Oxford 3120 temperature controller. Protein concentrations of the EPR samples were determined spectrophotometrically at 280 nm ($\epsilon = 3.6 \times 10^5$ M^{-1} cm^{-1}; Song & Frey, 1991). Spin concentrations were estimated by double integration of EPR signals, using copper perchlorate standards at 10 μW and 100 μW microwave power at 10 K. Power saturation studies were performed at 10 K and analyzed by the method of Beinert and Orme-Johnson (1967). Samples of enzyme in standard isolation buffer were prepared by adding the sample anaerobically to an EPR tube and freezing in liquid nitrogen.

Samples of enzyme oxidized with ferricyanide were generated after the dithiothreitol was removed by desalting anaerobically using a 4-mL Sephadex G-25 spin column

(Penefsky, 1978) equilibrated with 30 mM Tris-HCl at pH 8.0 containing 0.1 mM L-lysine. After desalting, the enzyme was oxidized by adding 30 μL of a 2 mM potassium ferricyanide solution to 270 μL of desalted enzyme (27 μM enzyme) and allowing the solution to equilibrate for 2 min. Alternative oxidative methods were (a) simply gel filtering aerobically using a 4-mL Sephadex G-25 spin column or b) anaerobically adding 30 μL of 20 mM potassium ferricyanide to 270 μL of enzyme (65 μM) in the standard isolation buffer and allowing the solution to equilibrate for 10 min.

For studies of the time course of reductive activation, samples were prepared using a direct volume scale-up of the concentrated activation procedure described above. The activity of a sample held at 37 °C in the activation solution for a given time was determined by transferring 3 μL into an assay mixture just prior to transferring the balance of the sample into an EPR tube and freezing with liquid nitrogen. The activities of the 3-μL aliquots were determined using the concentrated assay procedure of Ballinger et al. (1992). Less than 1.5 min elapsed from the time of the removal of the 3-μL aliquot, to the start of the activity assay and the freezing of the sample.

EPR Spectral Simulation. Spectral simulations were computed by numerical integration of the expression (Hecht, 1967)

$$\frac{dF}{dH} = \int_0^{2\pi}\int_0^{\pi}\frac{dG}{dH}\sin\theta\, d\theta\, d\phi \qquad (1)$$

where G is a Gaussian line shape function centered at a field position H_r (calculated resonance field).

$$H_r = \frac{h\nu}{g(\theta,\phi)\,\beta} \qquad (2)$$

In eq 2

$$g(\theta,\phi) = (g_{zz}^2\cos^2\theta + g_{xx}^2\sin^2\theta\cos^2\phi + g_{yy}^2\sin^2\theta\sin^2\phi)^{1/2}$$

RESULTS

Purification of Lysine 2,3-Aminomutase. In order to maximize the cobalt occupancy of the divalent metal ion binding site, bacteria were grown and the enzyme was purified by a modification of the best procedure described by Petrovich et al. (1991). To minimize the loss of cobalt from the protein in the course of purification, 10 μM $CoCl_2$ was included in the purification buffers, except for the elution buffer used for the last column chromatographic step (Q-Sepharose). Supplementation with $CoCl_2$ enhanced the cobalt content of the enzyme and increased the specific activity. Samples of enzyme purified in this way contained 5.1 ± 0.3 mol of cobalt and 10.1 ± 0.5 mol of iron/mol of enzyme (hexamer), and they exhibited specific activities of 32 ± 2 IU·(mg of protein)$^{-1}$, the highest activity reported to date. These preparations also contained 1.1 ± 0.2 mol of zinc and 0.25 ± 0.07 mol of copper/mol of hexameric enzyme. These samples extended the positive correlation between cobalt occupancy of the binding site for divalent metal ions and enzymatic activity. The best sample prepared by Petrovich et al. (1991) contained 3.6 mol of cobalt·mol^{-1} and exhibited a specific activity of 23 IU·(mg of protein)$^{-1}$. In these samples, the remainder of divalent metal binding sites were occupied by zinc and copper, which did not appear to support the enzymatic activity. The enzyme used in most of the present experiments contained significantly more cobalt and exhibited significantly higher activity than earlier preparations of lysine 2,3-aminomutase.

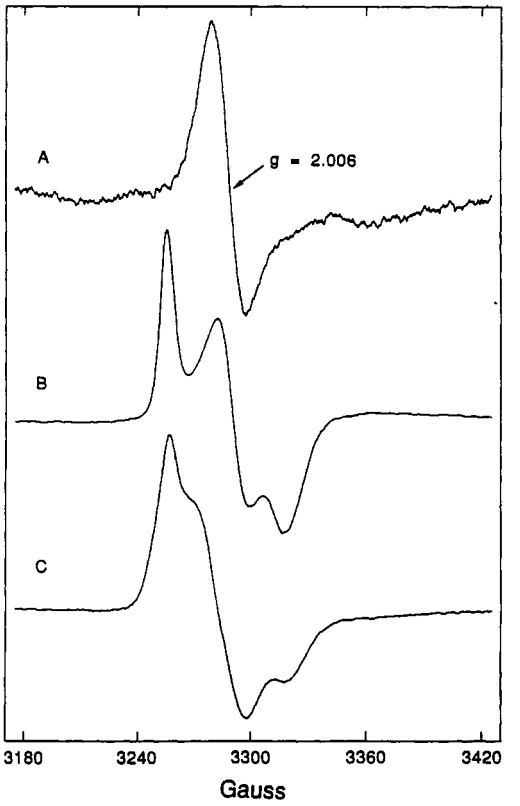


FIGURE 1: EPR spectra of lysine 2,3-aminomutase in the $g = 2$ region at 10 K. The enzyme samples in standard isolation buffer were transferred anaerobically to EPR tubes, which were sealed with rubber septa and frozen in liquid nitrogen. Spectra were recorded at 9.24 GHz, with a modulation amplitude of 5 G at 500 μW power. Receiver gains varied and are given below. (A) The enzyme concentration was 66 μM, it contained 12.5 mol of Fe/mol, and it had a specific activity of 20.4 ± 2 IU·(mg of protein)$^{-1}$. The receiver gain was 8 × 10^5. (B) The enzyme concentration was 61 μM, it contained 9.4 mol of Fe/mol, and it had a specific activity of 32 ± 2 IU·(mg of protein)$^{-1}$. The receiver gain was 1.25 × 10^5. (C) The enzyme concentration of 76 μM, it contained 13.2 mol of Fe/mol, and it had a specific activity of 23 ± 2 IU·(mg of protein)$^{-1}$. The receiver gain was 2.5 × 10^5.

The activity of this enzyme has also been shown to be positively correlated with the amount of iron and sulfide bound to the enzyme. The specific enzymatic activity reaches a maximum at the maximum ion level of approximately 12 mol·mol^{-1} (hexamer) of iron and of sulfide. The samples of enzyme used in the present study contained iron and sulfide at the levels of 9–13 mol·mol^{-1} (hexamer).

EPR Spectra of Lysine 2,3-Aminomutase. The presence of equimolar amounts of Fe and S^{2-} and the long wavelength absorbances in the published ultraviolet–visible spectrum of lysine 2,3-aminomutase are consistent with the presence of [Fe-S] clusters (Petrovich et al., 1991). The existence of [Fe-S] clusters in an enzyme may be verified by the appearance of signals in the EPR spectrum at 10 K (Orme-Johnson & Orme-Johnson, 1982). The EPR spectra (at 10 K) of three samples of lysine 2,3-aminomutase are shown in Figure 1. All samples of purified lysine 2,3-aminomutase exhibit EPR signals in the $g = 2$ region at 10 K, but the signal patterns vary from sample to sample, presumably owing to slight variations in the purification conditions. Spectra A and B in Figure 1 represent the extremes in the diversity of signal patterns for enzyme samples prepared as described in the preceding section. Spectrum B is typical of those for samples purified under strictly anaerobic conditions. Spectrum C is typical of samples that had been briefly exposed to air at some

point in the course of purification. The signals in Figure 1 represent between 0.2 and 1.5 spins per hexamer of enzyme. All three samples used in Figure 1 also exhibited a small EPR signal at $g = 4.3$ (not shown) characteristic of the presence of Fe(III). As estimated from the peak heights, the $g = 4.3$ signal in sample C was at least eight times larger than that from sample A and 100 times larger than that from sample B.

The spectrum of the sample in Figure 1A is slightly asymmetric and has a center g value of 2.006, and it is unchanged at temperatures up to 77 K. A study of the effects of power on this spectrum at 10 K reveals that it is subject to inhomogeneous broadening with a $p_{1/2}$ value of 550 ± 100 μW (data not shown). These EPR properties are consistent with those of an organic radical, and spectrum A in Figure 1 is designated the radical spectrum. Treatment of the sample with the radical quenching agent hydroxyurea or the spin trap DMPO does not alter the spectrum, which indicates that the radical is sequestered in the protein and inaccessible to these reagents. Equilibration of the enzyme for 3 h in 70% D_2O causes only a slight sharpening of the spectrum (1.2–1.5 G). Therefore, no readily exchangeable protons have strong hyperfine coupling to the unpaired electron. Any chemically exchangeable protons associated with a sequestered radical would not have undergone exchange under the conditions of this experiment. The absence of observable hyperfine splitting in the radical spectrum precludes a specific assignment of the species from which it originates.

Increasing the temperature at which the EPR spectrum is acquired dramatically alters the spectra for samples B and C in Figure 1, both of which revert to the spectrum of the radical at temperatures above 35 K. The effect of temperature on the spectrum of a sample similar to that of Figure 1B is shown in Figure 2. At higher temperatures, the outer features of the spectrum are broadened and attenuated, while the central features appear to be unaffected. At temperatures approaching 35 K the outer features disappear, leaving a spectrum (Figure 2A) that is very similar to that of the radical (Figure 1A). This behavior is consistent with the presence of two or more components in the spectrum acquired at 10 K, one of which is the radical spectrum.

The spectrum of a second component in the sample giving spectrum D in Figure 2 can be obtained by subtracting the radical spectrum (Figure 1A). The difference spectrum is shown in Figure 3A and is approximated in simulations with $g_{xx} = 2.027$, $g_{yy} = 2.007$, and $g_{zz} = 1.99$ (Figure 3B). The outer component of the difference spectrum ($g = 2.027$, 3258 G) undergoes power saturation at 10 K in a homogeneous manner, with a $p_{1/2}$ of 15 ± 2 mW. The properties of the difference spectrum are consistent with an [Fe-S] cluster, and it is designated the [Fe-S] spectrum.

To determine if the [Fe-S] spectrum originates from a reduced cluster, a sample of the enzyme in standard isolation buffer was treated with sodium dithionite (final concentration of 10 mM). This treatment had no effect on the low-temperature EPR spectrum of the enzyme in the region of $g = 2$. Because the [Fe-S] clusters may not have been accessible to dithionite, a second sample was treated with 10 mM sodium dithionite together with 6 μM methyl viologen as an oxidation–reduction mediator for the sodium dithionite. This treatment led to the loss of the EPR spectrum of the enzyme (data not shown). The results from reduction with sodium dithionite indicate that the spectrum of the [Fe-S] cluster does not originate with a reduced cluster; instead, the enzyme contains a cluster that is subject to reduction.

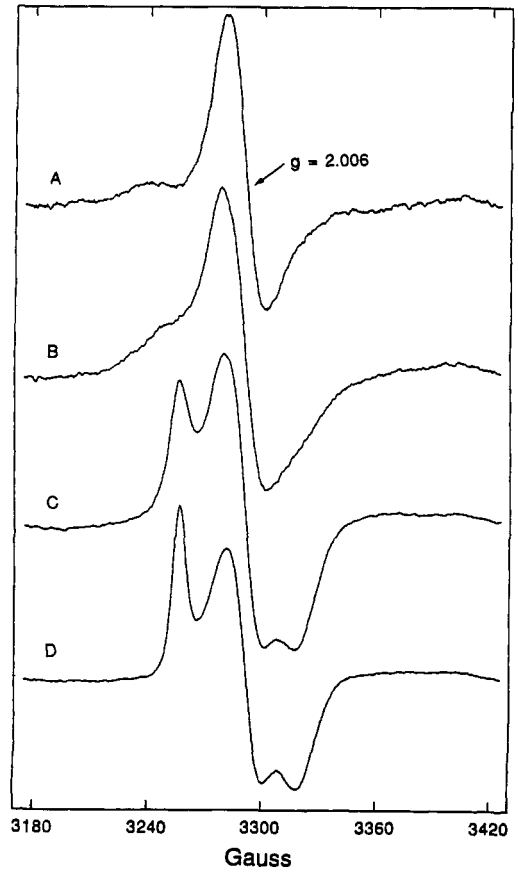


FIGURE 2: Effect of temperature on the EPR spectrum of lysine 2,3-aminomutase. The enzyme concentration was 92 μM, it contained 10.6 mol of Fe/mol, and it had a specific activity of 32 ± 2 IU·(mg of protein)$^{-1}$: (A) 35 K; (B) 25 K; (C) 15 K; (D) 10 K.

Spectrum C in Figure 1 is more complex than spectra A and B. Spectrum C consists of contributions from three components, one of which is the [Fe-S] cluster. To explain spectrum C of Figure 1, it is necessary to consider the effects of oxidation on the low-temperature EPR spectrum of lysine 2,3-aminomutase. The low-temperature EPR spectrum in Figure 4A is that of a sample of lysine 2,3-aminomutase that had been separated from dithiothreitol by passage through a Sephadex G-25 column and oxidized by treatment with potassium ferricyanide. The spectrum in Figure 4A is rhombic, as verified by the simulation in Figure 4B. The spectrum of Figure 4A persists but is broadened at 35 K and cannot be seen at 77 K; it undergoes power saturation at 10 K in a homogeneous fashion ($p_{1/2}$ value, 10 ± 3 mW). This spectrum can also be generated by exposing the enzyme to air or by adding potassium ferricyanide in a greater than 2-fold excess of the dithiothreitol present in the standard isolation buffer of the enzyme sample. The generation of the species giving the spectrum in Figure 4A by any method leads to at least a 25-fold increase in the intensity of the Fe(III) signal seen at $g = 4.3$.

The spectrum in Figure 4A is similar to those of iron–sulfur clusters of the type [3Fe-4S] (Beinert & Thomson, 1983), and this spectrum is designated the [3Fe-4S] spectrum. This spectrum appears to be generated by an oxidative degradation of the [Fe-S] cluster into a [3Fe-4S] cluster. Double integration of the [3Fe-4S] spectrum indicates that there are 2.5–3 spins/hexamer of enzyme.

Subtraction of the [3Fe-4S] spectrum from spectrum C in Figure 1 yields a difference spectrum that is similar to that of Figure 1B (data not shown). This spectrum consists of two

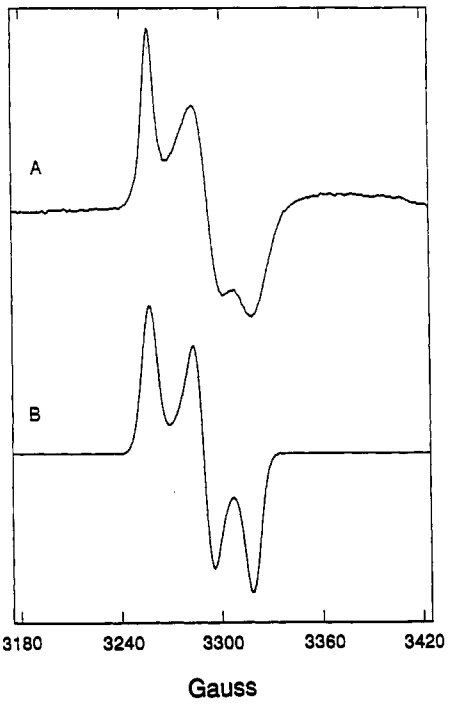


FIGURE 3: Difference spectrum of lysine 2,3-aminomutase at 10 K. (A) The difference spectrum was generated by subtracting spectrum 1A from spectrum 2D using a scalar factor of 0.33. This scaling factor arises from a comparison of the amounts of the radical spectrum present in the spectra of the two samples taken at 77 K, correcting for the receiver gain differences in the two spectra taken at 10 K. The subtraction process also requires an intensity correction for the difference in microwave power used to obtain the two spectra in Figures 1 and 2. The residual spectrum is that of the [Fe-S] cluster ([4Fe-4S]$^{3+}$). (B) Simulation of the difference spectrum.

components, the radical spectrum and the [Fe-S] spectrum. Therefore, spectrum C in Figure 1 appears to consist of three components, the radical spectrum, the [Fe-S] spectrum, and the [3Fe-4S] spectrum.

It appears that the low-temperature EPR spectrum of the intact [Fe-S] cluster in lysine 2,3-aminomutase is the difference spectrum shown in Figure 3A. The EPR signals of two of the purified enzyme samples in Figure 1 (B and C) contain contributions from the [Fe-S] cluster, whereas sample A only contains the radical. Inasmuch as the enzyme samples in Figure 1 contain only 0.2–1.5 spins/hexamer, and the maximum number of spins contributed by the [3Fe-4S] clusters is 2.5–3/hexamer, the enzyme samples giving the spectra in Figure 1 must have contained variable amounts of EPR-silent [Fe-S] clusters. As shown in the following section, dihydrolipoate reduces the EPR-visible [Fe-S] clusters to an EPR-silent form. The simplest interpretation of the spin concentrations found for different samples of purified enzyme is that part of the intact [Fe-S] clusters are in the reduced (EPR-silent) form and part are in higher oxidation state that exhibits the difference spectrum in Figure 3A, that of the intact [Fe-S] cluster.

The [Fe-S] spectrum is unlike the spectra for known [Fe-S] clusters. The following facts indicate but do not prove that it is a [4Fe-4S] cluster: (a) The g values of the spectrum and the fact that it is reduced to an EPR-silent form are consistent with a [4Fe-4S]$^{3+}$ cluster (Orme-Johnson & Orme-Johnson, 1982); (b) Oxidation of the enzyme by ferricyanide or oxygen transforms the cluster into one that exhibits an EPR spectrum typical of [3Fe-4S] clusters. This process is accompanied by an increase in the Fe(III) signal at $g = 4.3$.

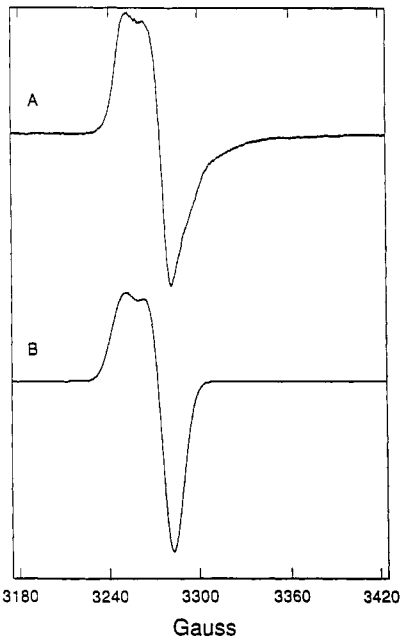


FIGURE 4: EPR spectrum of oxidized lysine 2,3-aminomutase at 10 K in the $g = 2$ region. (A) The enzyme was prepared by removing the dithiothreitol by centrifugation of the sample through a 4-mL Sephadex G-25 spin column (Penefsky, 1978) equilibrated with 30 mM Tris-HCl, pH 8.0, containing 0.1 mM lysine under anaerobic conditions. After the dithiothreitol was removed, the enzyme was oxidized by adding 30 μL of a 2 mM potassium ferricyanide solution to 270 μL of desalted enzyme. The final enzyme concentration was 27 μM. (B) Simulation of the spectrum of the oxidized enzyme. The parameters used in the simulation were $g_{xx} = 2.032$, $g_{yy} = 2.015$, and $g_{zz} = 2.0125$.

Effects of Thiols on the EPR Signals of Lysine 2,3-Aminomutase. Purified lysine 2,3-aminomutase is partially activated by the addition of *S*-adenosylmethionine. Preliminary incubation with a thiol such as dihydrolipoate or glutathione for 4–5 h at 37 °C leads to an enzyme that can be fully activated by addition of *S*-adenosylmethionine (Chirpich et al., 1970; Moss & Frey, 1987). The spectra of both the radical and the [Fe-S] cluster associated with the purified enzyme are removed by the reductive activation process. The loss of the [Fe-S] spectrum under the conditions of reductive incubation with 15 mM dihydrolipase indicates that its reduction potential is more positive than −370 mV. From the fact that the [Fe-S] cluster is partially oxidized in the isolation buffer, which contains 1 mM dithiothreitol, E_0'(pH 8.1) = −366 mV (Cleland, 1964), we estimate that the reduction potential should not be more positive than −338 mV. By these criteria, the reduction potential should lie between −338 and −370 mV.

The removal of both the [Fe-S] spectrum and the radical spectrum by reductive activation indicated that one or both of these species may be associated with an inactive form (or forms) of the enzyme. To investigate this possibility, the course of the reductive activation at 37 °C was monitored by activity measurements and EPR spectroscopy as a function of time. The EPR spectra were monitored at both 10 K and 35 K (data not shown). As shnown in Figure 5, the total signal monitored at 10 K in the region of $g = 2$ decreased dramatically within 1 h and was completely abolished after 4 h (less than 1% remaining of the starting signal intensity). Inspection of the spectra taken at 10 K as a function of time indicated that the composition changed during the reductive activation process. The [Fe-S] spectrum appeared to be disappearing at a faster rate than the radical spectrum. In order to determine whether

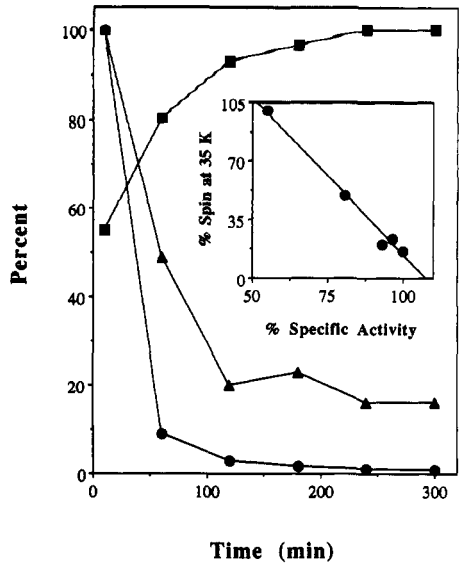


FIGURE 5: Correlation between the loss of EPR signals and enzyme activation in the course of the preliminary reductive incubation process. Samples were prepared by adding 1080 μL of enzyme in standard isolation buffer to 720 μL of a concentrated activation stock (9 mM dihydrolipoic acid, 2.4 mM Fe(II), 0.96 mM PLP, and 100 mM Tris-HCl, pH 8.0) and then transforming aliquots of 300 μL into six 500-μL Eppendorf microfuge tubes to be incubated at 37 °C. The final concentration of enzyme in each sample was 37 μM. After the incubation time indicated, the sample activities were measued using the concentrated assay of Ballinger et al. (1992) prior to being anaerobically transferred to an EPR tube and frozen with liquid nitrogen. Symbols: (●) percent of initial signal in the EPR spectrum obtained at 10 K (minus the 0.9% residual signal at infinite time) during the course of the reductive incubation process (the value at 10 min was set at 100%); (▲) percent of initial signal in the EPR spectrum taken at 35 K during the course of the reductive incubation process (the value at 10 min was set at 100%); (■) percent of the maximum specific activity attained at various times in the reductive incubation process (the value after 300 min was set at 100%). (Inset) Percent signal at 35 K as a function of the percent of maximal specific activity.

this was the case, the radical was monitored without interference from the [Fe-S] cluster by observing the EPR spectra at 35 K. Double integration of the radical spectrum as a function of time showed that the radical signal disappeared less rapidly than that of the [Fe-S] cluster (Figure 5).

Plots of the percent signal, monitored at 10 K and at 35 K as a function of time, and the percent activity versus time are shown in Figure 5. The shapes of the curves indicate that activation of the enzyme, the decrease in the radical signal measured at 35 K, and the loss of the total signal owing to the radical plus the [Fe-S] cluster measured at 10 K are all first-order processes. The rate constants for the increase in enzymatic activity and the decrease in the radical concentration are $(1.4 \pm 0.2) \times 10^{-2}$ min^{-1} and $(1.8 \pm 0.4) \times 10^{-2}$ min^{-1}, respectively. These rate constants are similar, in fact the same within experimental error. The rate constant for the loss of the signals for the radical plus the [Fe-S] cluster measured at 10 K is $(2.7 \pm 0.3) \times 10^{-2}$ min^{-1}. The major part (96%) of the signal observed at 10 K is due to the [Fe-S] cluster, which undergoes reduction with a rate constant that is significantly larger than that for the activation of the enzyme.

Reductive Activation of the [3Fe-4S] Enzyme. The species of [Fe-S] cluster that exhibits the [3Fe-4S] EPR spectrum is produced in the oxidation of the enzyme by air or ferricyanide in a process that is accompanied by an increase in the signal at $g = 4.3$. The enzyme containing this form of the partially disaggregated cluster retains its capacity to undergo reductive activation. A sample of lysine 2,3-aminomutase containing 12.5 mol of Fe, 12.0 mol of S^{2-}, and 3.6 mol of Co per molecule (hexamer) exhibited a specific activity of 20.5 ± 2 IU·(mg of protein)$^{-1}$ and an EPR spectrum similar to that in Figure 1B. Oxidation of the enzyme by passage through a Sephadex G-25 column (4 mL) under aerobic conditions converted the [Fe-S] cluster into the species that exhibits the [3Fe-4S] spectrum. Reductive activation of this sample using the dilute activation procedure restored 89% of its original activity.

DISCUSSION

The low-temperature EPR spectra confirm the presence of [Fe-S] clusters in lysine 2,3-aminomutase. The clusters exist in two oxidation states, an oxidized state exhibiting an EPR signal that can be detected at temperatures lower than 35 K and a reduced state that is EPR-silent. The g values and temperature dependence of the EPR spectrum are consistent with clusters of the type [4Fe-4S] (Orme-Johnson & Orme-Johnson, 1982).

We recently postulated that lysine 2,3-aminomutase might contain either six clusters of the type [2Fe-2S] or three clusters of the type [4Fe-4S], based on analyses for iron and sulfide and the fact that the enzyme is a hexamer of identical subunits (Petrovich et al., 1990). We favored the interpretation that the enzyme contains [4Fe-4S] clusters, because cross-linking experiments had shown that the hexameric enzyme is assembled from dimers (Song & Frey, 1991). Assembly of a hexamer from three dimers could take place if each subunit in a dimer contributes ligands to a shared [Fe-S] cluster. Three such clusters would account for the iron and sulfide content of the enzyme if it contained [4Fe-4S] clusters. Precedent for [4Fe-4S] clusters bridging two protein subunits, each of which contributes ligands to the cluster, is provided by the nitrogenase Fe-protein from *Azotobacter vinelandii* (Gillum et al., 1977; Hausinger & Howard, 1983).

Further evidence supporting the identification of [4Fe-4S] clusters is provided by the oxidation of the enzyme by air or ferricyanide, leading to a species with an EPR spectrum characteristic of [3Fe-4S] clusters. The spin content of the oxidized enzyme shows that it contains 2.7 ± 0.2 spins per molecule. The oxidized enzyme can be reactivated under the assay conditions, which include preliminary incubation with Fe(II) and a reducing system, and it presumably leads to the reconstitution of the clusters. The most obvious interpretation of these facts is that the enzyme contains three [4Fe-4S], which undergo disaggregation to [3Fe-4S] clusters upon oxidation. The purified enzyme appears to contain clusters of the type $[4Fe\text{-}4S]^{3+}$ and the EPR-silent $[4Fe\text{-}4S]^{2+}$, such that the net spin content of the enzyme is less than three spins per molecule, generally about 1 spin. Reductive activation transforms all of the clusters into the latter type, and oxidation of the enzyme transforms them into [3Fe-4S] clusters.

Earlier preparations of lysine 2,3-aminomutase contained much less iron and sulfide than that corresponding to three [3Fe-4S] clusters, and these samples could not be fully activated by preliminary reductive incubation with Fe(II) (Petrovich et al., 1991). These preparations presumably had lost part of the [Fe-S] clusters and could not be reconstituted under the activation conditions.

The organic radical associated with the purified enzyme cannot be identified from the available information. The signal lacks resolved hyperfine structure of the type reported for protein radicals, such as the glycine radical in pyruvate formate lyase (Wagner et al., 1992) and the tyrosyl radicals that have been identified in ribonucleotide reductase (Reichard & Ehrenberg, 1983; Bender et al., 1989), prostaglandin H

synthase (Karthein et al., 1988), photosystem II (Debus et al., 1988), and galactose oxidase (Whittaker & Whittaker, 1990; Babcock et al., 1992). A tyrosyl radical cannot be ruled out, however, because a singlet resonance in a tyrosyl radical is possible when the dihedral angles relating the methylene protons to the axis perpendicular to the aromatic ring are both greater than 45° (Lassman et al., 1991). For example, the singlet EPR signal observed in prostaglandin H synthase reconstituted with Fe(III)–protoporphyrin IX is nearly isotropic and centered at $g = 2.004$. The stable tryptophan radical in cytochrome *c* peroxidase compound ES gives a complex multiplet EPR spectrum unlike that of the radical spectrum in lysine 2,3-aminomutase (Sivaraja et al., 1989; Hori & Yonetani, 1985). The significance of the radical in lysine 2,3-aminomutase is not known. The fact that it is quenched by the reductive activation conditions at the same rate that the enzyme is activated implies but does not prove that it may be an artifact of purification. The origin of this radical is unknown.

In addition to the [Fe-S] cluster, lysine 2,3-aminomutase contains a second metallic cofactor, cobalt, which is required for maximal activity. The relationship between cobalt and the [Fe-S] clusters is not known, but the EPR spectra at 10 K rule out the possibility that the enzyme might contain a mixed Co–Fe–sulfide complex. Such clusters can be generated from the [4Fe-4S] cluster in ferredoxin II from *Desulfovibrio gigas* by oxidation to [3Fe-4S] and reconstitution with $Co(NO_3)_2$ in the presence of dithiothreitol (Moura et al., 1986). The mixed complex [Co-3Fe-4S] gives a rhombic EPR spectrum at 40 K, with $g_{zz} = 1.98$, $g_{yy} = 1.94$, and $g_{xx} = 1.82$. The presence of Co(II) in this complex is evident in the EPR spectrum, which exhibits resolved ^{59}Co hyperfine splitting with a coupling constant of 44 G. The absence of ^{59}Co hyperfine splitting in the EPR spectrum of the [Fe-S] cluster in lysine 2,3-aminomutase rules out the possibility that cobalt and iron coexist within a mixed metal–sulfur cluster.

Lysine 2,3-aminomutase is exceptional among aminomutases in that it utilizes *S*-adenosylmethionine in place of adenosylcobalamin to mediate hydrogen transfer (Moss & Frey, 1987; Baraniak et al., 1989). Most aminomutases, including D-ornithine 4,5-aminomutase, β-lysine mutase, and D-lysine mutase are activated by adenosylcobalamin (Somack & Costilow, 1973; Baker et al., 1973; Morley & Stadtman, 1970). In the B_{12}-dependent aminomutases, adenosylcobalamin is the source of the 5′-deoxyadenosyl radical, the hydrogen atom abstracting species that mediates hydrogen transfer in these reactions. In the case of lysine 2,3-aminomutase, the cofactor *S*-adenosylmethionine lacks the organometallic properties of adenosylcobalamin and cannot, by itself, generate the 5′-deoxyadenosyl radical. We postulate that lysine 2,3-aminomutase is activated through a reaction of *S*-adenosylmethionine with one or both of the metallic cofactors to form an adenosyl-cofactor, which leads to the reversible formation of the 5′-deoxyadenosyl radical (Moss & Frey, 1987; Petrovich et al., 1991). By analogy with adenosylcobalamin, it is reasonable to expect the adenosyl-cofactor to be an adenosyl-cobalt complex. We cannot rule out the possibility of an adenosyl-[Fe-S] cluster, either as the adenosyl-cofactor or as an intermediate in the formation of an adenosyl-cobalt complex; however, currently available data do not provide any evidence for an adenosyl-[Fe-S] cluster.

While the function of the [Fe-S] clusters in lysine 2,3-aminomutase remains to be established, it seems likely that they somehow facilitate the activation of the enzyme by *S*-adenosylmethionine. An obvious role for [Fe-S] clusters would be to participate in the formation of the putative adenosyl-cofactor that provides for the reversible formation of the 5′-deoxyadenosyl radical. If the adenosyl-cofactor is an adenosyl-cobalt complex, the reduced [Fe-S] cluster might convert a small fraction of the Co(II) in the enzyme to a Co(I) complex by electron transfer, and Co(I) could be captured as an adenosyl-Co(III) complex by alkylation with *S*-adenosylmethionine at the active site according to

$$[4Fe\text{-}4S]^{2+} + Co(II) \rightleftharpoons [4Fe\text{-}4S]^{3+} + Co(I) \quad (3)$$

$$Co(I) + S\text{-adenosylmethionine} \rightleftharpoons \text{adenosyl-Co(III)} + \text{Methionine} \quad (4)$$

This process would be analogous to the methylation of methionine synthase by *S*-adenosylmethionine (Banerjee, et al., 1990), in which the methyl group of *S*-adenosylmethionine is transferred to the cobalt of vitamin B_{12} rather than the adenosyl group. Alternatively, *S*-adenosylmethionine may adenosylate the reduced [Fe-S] cluster, and the resulting adenosyl-[Fe-S] complex may transiently generate the 5′-deoxyadenosyl radical, which is captured and stabilized by reaction with Co(II) in a nearby binding site according to

$$[4Fe\text{-}4S]^{2+} + S\text{-adenosylmethionine} \rightleftharpoons \text{adenosyl-}[4Fe\text{-}4S]^{2+} + \text{methionine} \quad (5)$$

$$\text{adenosyl-}[4Fe\text{-}4S]^{2+} \rightleftharpoons [4Fe\text{-}4S]^{3+} + \text{adenosyl-5′-}CH_2^{\bullet} \quad (6)$$

$$\text{adenosyl-5′-}CH_2^{\bullet} + Co(II) \rightleftharpoons \text{adenosyl-Co(III)} \quad (7)$$

The identification of the putative adenosyl-cofactor and the role of [Fe-S] clusters and cobalt in the activation of lysine 2,3-aminomutase are under continuing investigation in this laboratory.

ACKNOWLEDGMENT

We thank Professor Helmut Beinert for hosting us in his laboratory to obtain a preliminary low-temperature EPR spectrum of the iron–sulfur cluster in lysine 2,3-aminomutase, for advice on the interpretation of the early spectra, and for valuable comments on early drafts of this paper.

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