Biochemistry

© Copyright 1992 by the American Chemical Society Volume 31, Number 4 February 4, 1992

Accelerated Publications

An Organic Radical in the Lysine 2,3-Aminomutase Reaction†

Marcus D. Ballinger, George H. Reed,* and Perry A. Frey*

Institute for Enzyme Research, Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53705

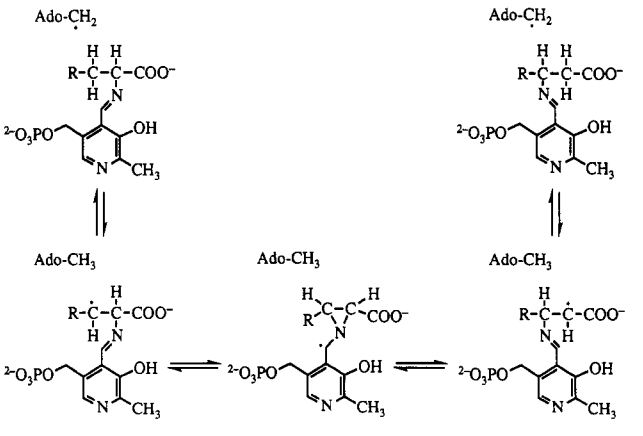
Received November 15, 1991; Revised Manuscript Received December 9, 1991

ABSTRACT: Lysine 2,3-aminomutase from *Clostridium* SB4 has been studied by electron paramagnetic resonance (EPR) spectroscopy at 77 K. Although the reaction catalyzed by this enzyme is similar to rearrangements catalyzed by enzymes requiring adenosylcobalamin, lysine 2,3-aminomutase does not utilize this cofactor. The enzyme instead contains iron–sulfur clusters, cobalt, and pyridoxal phosphate and is activated by *S*-adenosylmethionine. Subsequent to a reductive incubation procedure that is required to activate the enzyme, EPR studies reveal the appearance of an organic radical signal ($g = 2.001$) upon addition of both L-lysine and *S*-adenosylmethionine. The radical signal is complex, having multiple hyperfine transitions. The total radical concentration is proportional to enzyme activity and decreases in parallel with the approach to chemical equilibrium between α-lysine and β-lysine. The signal changes over the time course of the reaction in a way that suggests the presence of more than one radical species, with different relative proportions of species in the steady state and equilibrium state. Isotopic substitution experiments show that unpaired spin density resides on the molecular framework of lysine and that solvent-exchangeable protons do not participate in strong hyperfine coupling to the radical. The results indicate that lysine radicals participate in the rearrangement mechanism.

Lysine 2,3-aminomutase catalyzes the first step in lysine metabolism in *Clostridium*, the conversion of L-α-lysine to L-β-lysine (Chirpich et al., 1970; Stadtman, 1973). The enzyme is a hexamer of apparently identical subunits with an overall molecular weight of 285 000, and it contains iron–sulfur clusters, as well as high-spin cobalt(II) and pyridoxal phosphate (Chirpich et al., 1970; Petrovich et al., 1991; Song & Frey, 1991). The protein loses activity after purification, and reactivation requires incubation with a sulfhydryl compound, iron(II), and pyridoxal phosphate, followed by addition of *S*-adenosylmethionine—all under strictly anaerobic conditions.

Mechanistic studies have led to the working hypothesis that the activation of lysine 2,3-aminomutase requires the reaction of a reduced metal cofactor with *S*-adenosylmethionine, leading to an adenosyl-cofactor species (Moss & Frey, 1990; Petrovich et al., 1991). This species would then reversibly

Scheme I



generate a 5′-deoxyadenosyl radical. The 5′-deoxyadenosyl radical is postulated to initiate a radical rearrangement by abstracting a hydrogen from carbon 3 of the imine formed

† This work was supported by Grant DK28607 from the National Institute of Digestive and Kidney Diseases (to P.A.F.), Grant GM35752 from the National Institute of General Medical Sciences (to G.H.R.), and Molecular Biophysics Training Grant GM08293-02 (to M.D.B.).

* Address correspondence to these authors at the Institute for Enzyme Research, University of Wisconsin, 1710 University Ave., Madison, WI 53705.

0006-2960/92/0431-949$03.00/0 © 1992 American Chemical Society

between α-lysine and PLP,[1] as shown in Scheme I. The resulting radical is further postulated to undergo a 1,2-imino radical rearrangement to the product radical, which abstracts a hydrogen atom from 5′-deoxyadenosine to form the product and regenerate the 5′-deoxyadenosyl radical. This hypothesis suggests a role for the 5′-deoxyadenosyl radical analogous to that for this species in adenosylcobalamin-dependent rearrangements (Frey, 1990).

Although the function of *S*-adenosylmethionine in mediating hydrogen transfer between carbon 3 and carbon 2 of lysine and β-lysine has been demonstrated (Moss & Frey, 1987; Baraniak et al., 1989; Kilgore & Aberhart, 1991), no direct experimental evidence for a radical mechanism has thus far been obtained. This paper reports the observation of EPR signals for organic radicals in aminomutase reaction mixtures. These EPR measurements show that the radicals are associated with enzyme activity and represent one or more intermediates on the reaction pathway.

Experimental Procedures

Purification of Lysine 2,3-Aminomutase. The enzyme was purified by following the procedure of Moss and Frey (1990), as amended by Petrovich et al. (1991). The purification was carried out within a Coy anaerobic chamber. The final step was ion-exchange chromatography through a 50-mL column of QAE-Sepharose. The purified enzyme was concentrated to ~50 mg/mL by vacuum dialysis in a collodion bag.

Isotopically Labeled Lysine. L-[3,3,4,4,5,5,6,6-$^2H_8$]Lysine (99% enrichment) was obtained from Merck.

Activation and Assay at High Enzyme Concentration. The activation and assay procedure outlined by Chirpich et al. (1970) was modified to optimize the specific activity at the higher enzyme concentrations required for EPR experiments. An important improvement was to perform the entire procedure in the anaerobic chamber. For a typical reduction and radiochemical assay, a stock mixture for the reductive incubation contained 80 mM Tris-HCl at pH 8.0, 6 mM sodium dithionite, 2 mM PLP, and 18 mM dihydrolipoate. Iron(II), which is required for activation at low enzyme concentrations (~200 nM) (Chirpich et al., 1970), was omitted from the activation mixtures because it gave no enhancement of specific activity at high (~100 μM) enzyme concentration. An aliquot (3 μL) of this solution was combined in a 0.5-mL microfuge tube with lysine 2,3-aminomutase (53 mg/mL, 12 μL) which was in the standard isolation buffer containing 30 mM Tris-HCl at pH 8.0 with 1 mM dithiothreitol, 0.01 mM PLP, and 0.1 mM L-lysine (Moss & Frey, 1990), and the mixture was incubated for 4 h at 36 °C. A 3-μL aliquot was then removed by a Hamilton syringe and injected into a cofactor–radiolabeled substrate mixture (12 μL), giving the following final concentrations of components: 60 mM Tris-HCl at pH 8.0, 20 mM sodium dithionite, 64 mM L-[U-^{14}C]lysine (0.021 μCi/μmol), and 1.2 mM *S*-adenosylmethionine, along with activation components carried over with reduced enzyme. The solution was quickly mixed by gentle hand vortexing, and the reaction was terminated at ~30 s by injection of 25 μL of 0.2 M formic acid. Radiolabeled α- and β-lysines were separated by paper electrophoresis, and the radioactivity was measured by scintillation counting as described in the original assay (Chirpich et al., 1970). Specific activities ranged from 5 to 10 IU mg^{-1}.

EPR Spectroscopy. Samples of reduced enzyme were prepared by scaling up the volumes in the reductive activation procedure described above. For a multiple-sample experiment, an activated enzyme mixture was prepared, and for each sample, an aliquot was withdrawn and added to a cofactor–substrate solution (nonradioactive) in a microfuge tube to give a final volume of 220 μL. The solution was then mixed and transferred to a 3-mm (i.d.) quartz EPR tube. After 1.5 min at room temperature, the tube was capped with a rubber septum and immersed in liquid nitrogen. For the sample in 77% ^2H_2O, the activated enzyme was diluted into a ^2H_2O solution of cofactor and substrate.

In the study of variable enzyme activity, aliquots were withdrawn from an activated enzyme mixture and subjected to both radiochemical and EPR assays. EPR samples were frozen in liquid nitrogen after 3-min reactions at room temperature.

For the studies of the radical signal intensity throughout the approach to chemical equilibrium, a 490-μL aliquot of reduced enzyme was initially added to the cofactor–substrate solution to give a final volume of 1.8 mL. The solution was mixed, and aliquots were withdrawn at various time intervals, transferred to EPR tubes, and frozen in liquid nitrogen.

EPR spectra were recorded at X-band on a Varian E3 spectrometer. A standard liquid nitrogen immersion dewar was used. The spectrometer was interfaced with an IBM AT microcomputer for data acquisition and analysis. Resolution enhancement of spectra was performed using the Fourier method described by Kauppinen et al. (1981). The Fourier transforms were deconvoluted with a Lorentzian, $\exp(2\pi\sigma|t|)$, where σ is the line width, and apodized with a squared triangular (Bartlett) window function (Kauppinen et al. 1981). Fourier transformation and deconvolution were carried out off-line with software written in Fortran. Spin concentrations were determined by double integration of spectra, and the integrals were referenced to that of a standard sample, Varian weak pitch (no. 90445002), which also served as a *g*-value (g = 2.0028) marker.

Results and Discussion

EPR Spectra of Reduced Enzyme Samples. In order to determine whether organic radicals are intermediates in the lysine 2,3-aminomutase reaction, we have examined various enzyme–cofactor–substrate mixtures by EPR at 77 K. The EPR spectra revealed the presence of organic radicals in solutions consisting of activated enzyme, *S*-adenosylmethionine, and lysine. In designing experiments, it was assumed that radical intermediates might be present in amounts representing only a small percentage of enzyme active sites in either the steady state or the equilibrium state of the reaction. Therefore, conditions were sought to maximize the concentration and activity of the aminomutase in test solutions. This objective presented problems, because initial experiments showed a trend toward decreasing specific enzymatic activity with increasing enzyme concentration. Furthermore, high concentrations of the enzyme naturally shorten the duration of the steady state. Modifications in the activation procedure (see Experimental Procedures) led to specific activities corresponding to about 50% of the maximum attainable with dilute enzyme, and it was possible to examine the steady state by using a high concentration of L-lysine.

Samples of the complete reaction mixture, including reduced enzyme, *S*-adenosylmethionine, and L-lysine, reproducibly exhibit an EPR signal centered at g = 2.001 (Figure 1D). A series of control experiments show that the EPR signal is associated with enzyme activity and is not a byproduct of the

[1] Abbreviations: EPR, electron paramagnetic resonance; PLP, pyridoxal 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; QAE, quaternary aminoethyl.

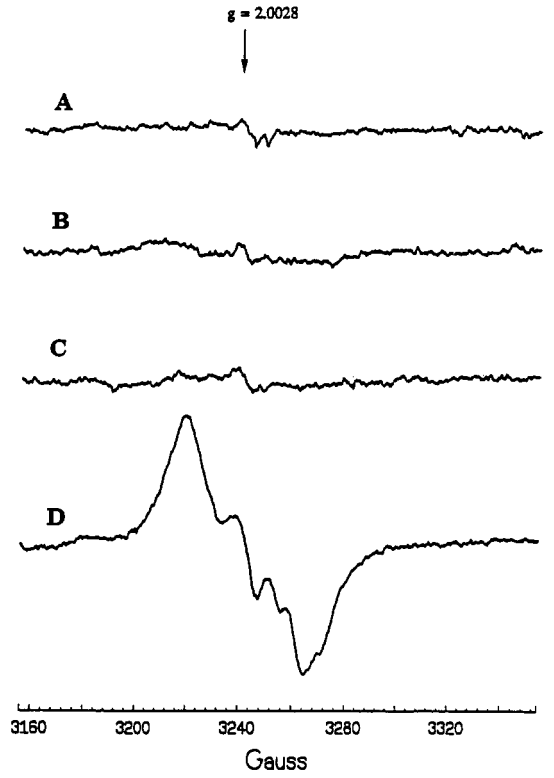


FIGURE 1: EPR spectra of reduced lysine 2,3-aminomutase at 77 K. All samples contained 31 μM enzyme (hexamer concentration). Spectra: (A) enzyme reduced in preparation for activation; (B) reduced enzyme plus 200 mM L-lysine; (C) reduced enzyme plus 1.2 mM AdoMet; (D) reduced enzyme plus 200 mM L-lysine and 1.2 mM AdoMet, frozen within 90 s, in the steady state. Instrument settings: scan range, 200 G; center field 3255 G; time constant, 1.0 s; scan time, 4 min; microwave frequency, 9.09 GHz; modulation frequency, 100 kHz; modulation amplitude, 3.2 G; microwave power, 2.5 mW; receiver gain, 5.0×10^5.

activation protocol. The spectrum does not appear in samples containing reduced enzyme alone (Figure 1A), reduced enzyme with added L-lysine (Figure 1B), or reduced enzyme with added *S*-adenosylmethionine (Figure 1C). Moreover, the spectrum is absent in samples containing otherwise complete reaction mixtures in which lysine 2,3-aminomutase was omitted or replaced with bovine serum albumin. Finally, the intensity of the EPR signal is negligible in spectra of samples of the complete reaction mixtures in which L-lysine is replaced with D-lysine (spectrum not shown).

The *g* value of 2.001, the appearance of partially resolved hyperfine structure, the relatively narrow widths of the signals, and the fact that the EPR signals of high-spin Co(II) and of the iron–sulfur centers in the enzyme are not observable at 77 K all indicate that these EPR signals are derived from an organic radical species. The multiplicity in the spectrum is characteristic of electron-nuclear spin hyperfine structure. These radical signals, however, behave somewhat anomalously with respect to spin–lattice relaxation, as the onset of saturation begins at power levels >5 mW at 77 K, and the signals are still present at power settings of >200 mW. This relaxation behavior could be caused by proximity of the radical center to a paramagnetic metal. The integrated intensity of the spectrum in Figure 1D corresponds to 2.9 μM spins, or 9.4% of the concentration of the oligomeric enzyme.

Dependence on Enzyme Concentration and Activity. The unpaired spin concentration in the $g = 2.0$ region depends on the concentration and activity of lysine 2,3-aminomutase, as shown in Figure 2. Data were obtained from samples which had been allowed to react long enough (3 min) for chemical

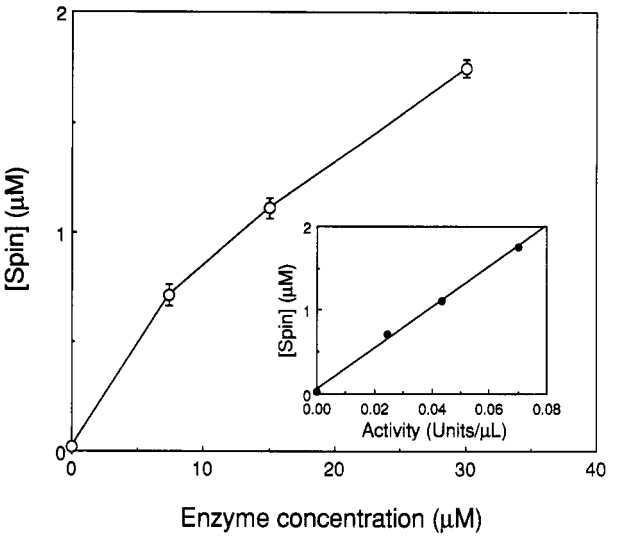


FIGURE 2: Unpaired spin concentration as a function of enzyme concentration. All samples contained 50 mM Tris-HCl at pH 8.0, 20 mM sodium dithionite, 80 mM L-lysine, and 1.2 mM AdoMet, in addition to components carried over from the reductive incubation along with the enzyme. Samples were frozen in liquid nitrogen after 3 min at room temperature, at which time the reactions were essentially at equilibrium. Each spin concentration is the average for two duplicate samples, with the range shown with bars. (Inset) Unpaired spin concentration as a function of enzyme activity. Activity was measured by radiochemical assay of the same reduced enzyme at concentrations identical to those used to generate EPR samples.

equilibrium between α-lysine and β-lysine to be established. It is important to note that the specific activity of the enzyme decreases slightly with increasing enzyme concentration. This effect is likely responsible for the slight curvature in the plot of unpaired spin concentration versus enzyme concentration, because the amount of unpaired spin increases linearly with total activity (Figure 2, inset). These data indicate that the radical is associated with enzyme that is actively turning over the substrate.

Relationships between the Radical Signal and the Ratio of α-Lysine/β-Lysine. Figure 3 shows the correlation between the integrated signal intensity and the approach to chemical equilibrium between α-lysine and β-lysine. As the ratio of β-lysine to α-lysine increases and approaches the equilibrium value, there is a corresponding decrease in the amount of unpaired spin to an equilibrium value. The approach to equilibrium and the decrease in integrated intensity follow the same time course. These observations suggest that the EPR signals arise from intermediate species whose concentration responds to the ratio of free L-β-lysine to L-α-lysine in bulk solution.

The appearance of the radical spectrum also changes during the course of the reaction. Spectra A and B in Figure 4 show the radical spectra from the earliest time point (22 s) and a time near equilibrium (4.5 min) in the experiment of Figure 3. The difference in the shapes of these signals is difficult to analyze from these raw spectra; however, the difference is more conspicuous after resolution enhancement. The resolution-enhanced spectra obtained are shown in Figure 4C,D. Analysis of the spectra in Figure 4C,D reveals that the hyperfine components in the early spectrum are still present in the late spectrum. However, spectra at later times show the gradual appearance of an additional feature at 3232 G (Figure 4D, vertical arrow), which is not discernible in spectra at early times. This suggests that the EPR spectrum is inhomogeneous and that the concentration of each radical varies differently with the ratio of α-lysine to β-lysine concentration. It is likely

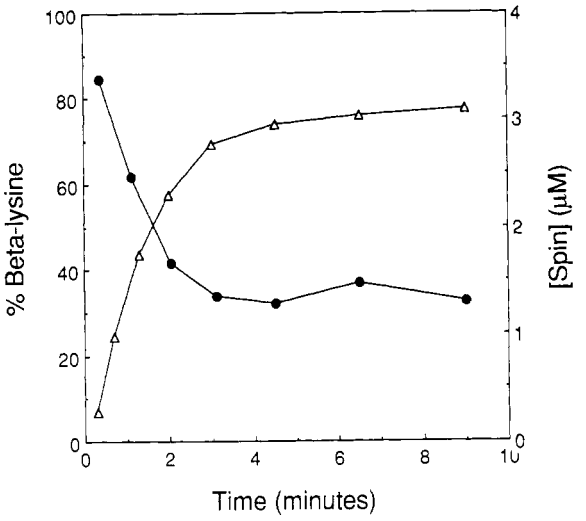


FIGURE 3: Correlation of the time course in the lysine 2,3-aminomutase reaction with a change in unpaired spin concentration. EPR samples were generated from a reaction containing 30 μM enzyme, 50 mM Tris-HCl at pH 8.0, 20 mM sodium dithionite, 200 mM L-lysine, and 1.2 mM AdoMet. Aliquots were withdrawn at various time intervals and frozen in liquid nitrogen. Spectra were then recorded, and unpaired spin concentrations were determined by double integration using a Varian weak pitch sample as a standard. The approach to chemical equilibrium was monitored by assays of L-α-[^{14}C]lysine and L-β-[^{14}C]lysine in solutions that were identical to those of the EPR samples. Symbols: (△) percent β-lysine of total lysines; (●) unpaired spin concentration.

that one of these species predominates in the steady state and is responsible for the relationship observed in Figure 3 between the total radical concentration and the approach to equilibrium between α-lysine and β-lysine.

Effects of Isotopic Substitutions on the Radical Signal. The correspondence between the properties of the radical signals and those expected of a reaction intermediate prompted an experiment with isotopically labeled substrate. The EPR spectrum obtained from a steady-state reaction mixture with L-[3,3,4,4,5,5,6,6-$^{2}H_8$]lysine (Figure 5B) exhibits marked changes from that of unlabeled lysine (Figure 5A). These spectral changes show that one or more protons at C3–C6 of the lysine are coupled to the predominant radical center. The improved spectral resolution that accompanies deuteration at C3–C6 is consistent with the smaller magnetogyric ratio of the deuteron. At the same time, the persistence of the predominant splitting pattern and of the width of this splitting manifold in the deuterated sample shows that protons from C3 to C6 of lysine are not responsible for the major hyperfine splitting in the spectrum. Rather, protons on these carbons are weakly coupled (scalar or dipolar) to the unpaired electron spin. Yet, even a weak hyperfine coupling from protons at C3–C6 virtually ensures that the predominant radical is centered on the molecular framework of lysine. The nearly "isotropic" appearance of the major hyperfine splitting suggests that this is a "π-type" radical species (Wertz & Bolton, 1986).

The effect of deuterium substitution in the solvent was also examined. The spectrum resulting from a reaction run in 77% $^{2}H_2O$ is shown in Figure 5C. The absence of any change in the splitting pattern indicates that solvent-exchangeable hydrogens, such as those of the amino groups of lysine, are not strongly coupled to the radical. The slight narrowing of the spectrum in 77% $^{2}H_2O$ is consistent with ^{2}H for ^{1}H exchange at positions where the ^{1}H is weakly coupled to the radical.

The presence of lysine-centered radicals during the reaction supports the radical mechanism postulated earlier (Moss & Frey, 1987, 1990; Baraniak et al., 1989, Petrovich et al., 1991).

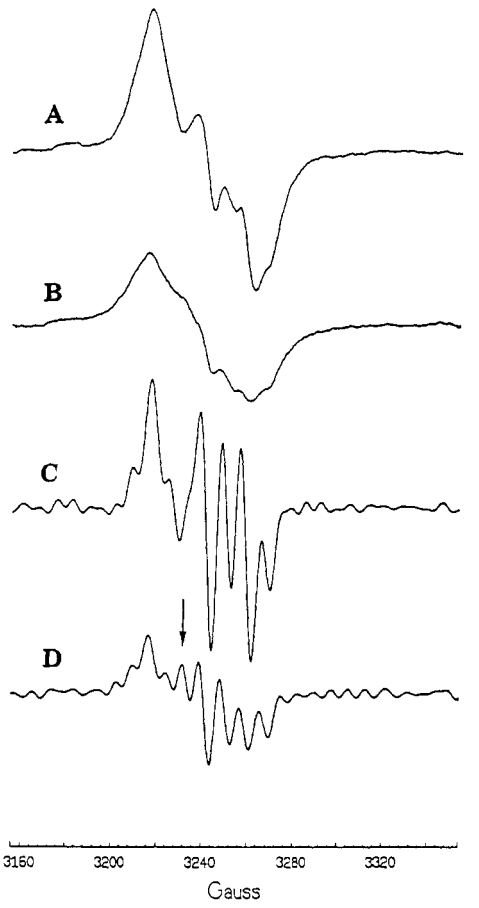


FIGURE 4: Change in the EPR spectrum from the steady state to the equilibrium state of the reaction. Spectra shown correspond to the 22-s time point and the 4.5-min time point samples of the experiment described in Figure 3 (spectra A and B, respectively). The spectra shown are the average of ten scans, using the same instrument settings described in Figure 1. Also shown are the same spectra after resolution enhancement (see Experimental Procedures) using a σ of 8.0 G and a truncation at 0.180 G^{-1} (spectra C and D).

In this mechanism, the reduced enzyme is activated by reaction of *S*-adenosylmethionine with a metal cofactor to generate a 5′-deoxyadenosyl-cofactor species. This species is proposed to function in a manner analogous to that of adenosylcobalamin, which undergoes homolytic cleavage of the Co–C bond to yield a 5′-deoxyadenosyl radical.

The 5′-deoxyadenosyl radical is postulated to initiate the rearrangement reaction by abstracting the 3-*pro-R* hydrogen of the imine formed between lysine and PLP, according to Scheme I. The resulting β-radical of lysine–PLP–imine is expected to undergo rearrangement to an azocyclopropinyl radical, which in turn undergoes rearrangement to the α-radical of β-lysine–PLP–imine. There is chemical precedent for this rearrangement (Han & Frey, 1990). The radicals observed in the experiments reported in this paper *may* correspond to those in Scheme I; however, the exact structures of the radicals observed in our experiments are not as yet known.

The structure of the adenosyl–metal cofactor utilized by lysine 2,3-aminomutase is as yet unknown; however, the direct involvement of the 5′-deoxyadenosyl moiety of *S*-adenosylmethionine in the hydrogen-transfer process and the production of 5′-deoxyadenosine and methionine have been demonstrated (Moss & Frey, 1987, 1990; Baraniak et al., 1989; Kilgore & Aberhart, 1991). The role of *S*-adenosylmethionine in generating the adenosyl cofactor of lysine 2,3-aminomutase may be related to the function of *S*-adenosylmethionine in activating

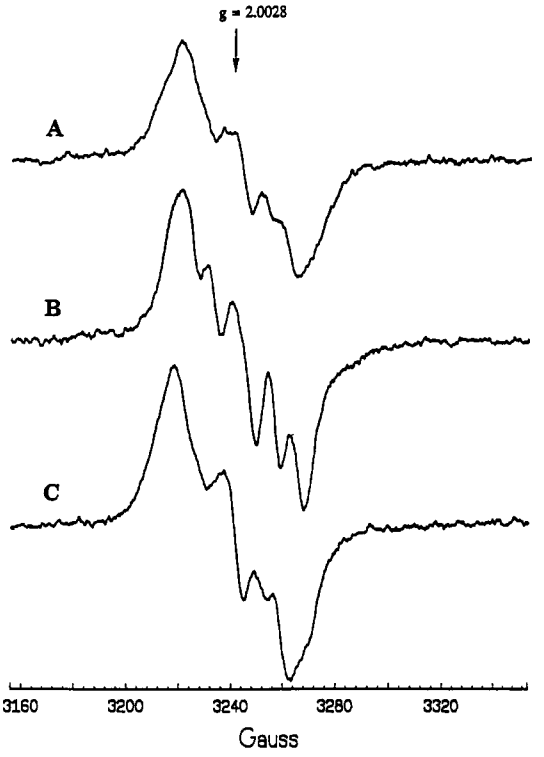


FIGURE 5: Effects of isotopic labeling in the substrate on the EPR spectrum. All samples were prepared as described in Figure 1D except for the listed isotopic substitutions. Spectra: (A) reaction with L-lysine; (B) reaction with L-[3,3,4,4,5,5,6,6-$^2H_8$]lysine (99% enrichment); (C) reaction in 77% ^2H_2O. Instrument settings were identical to those listed in Figure 1 except the receiver gain was 6.2×10^5.

pyruvate–formate lyase and the ribonucleotide reductase of *Escherichia coli* grown anaerobically (Knappe et al., 1984; Knappe & Sawyers, 1990; Eliasson et al., 1990). Pyruvate–formate lyase and anaerobic ribonucleotide reductase from *E. coli* are activated by activating enzymes, and the activating enzymes require *S*-adenosylmethionine. Both pyruvate–formate lyase and ribonucleotide reductase are thought to catalyze their reactions through radical mechanisms.

ACKNOWLEDGMENTS

We are grateful to David Latwesen for providing the software for resolution enhancement procedures and to Robert Petrovich for helpful discussions.

REFERENCES

Baraniak, J., Moss, M. L., & Frey, P. A. (1989) *J. Biol. Chem. 264*, 1357–1360.

Bolton, J. R., & Wertz, J. E. (1986) *Electron Spin Resonance*, pp 165–173, Chapman and Hall, London.

Chirpich, T. P., Zappia, V., Costilow, R. N., & Barker, H. A. (1970) *J. Biol. Chem. 245*, 1778–1789.

Eliason, R., Fontecave, M., Jornvall, H., Krook, M., Pontis, E., & Reichard, P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3314–3318.

Frey, P. A. (1990) *Chem. Rev. 90*, 1343–1357.

Han, O., & Frey, P. A. (1990) *J. Am. Chem. Soc. 112*, 8982–8983.

Kauppinen, J. K., Moffat, D. J., Mantsch, H. H., & Cameron, D. G. (1981) *Appl. Spectrosc. 35*, 271–276.

Kilgore, J. L., & Aberhart, D. J. (1991) *J. Chem. Soc., Perkin Trans. 1*, 79–84.

Knappe, J., & Sawyers, G. (1990) *FEMS Microbiol. Rev. 75*, 383–398.

Knappe, J., Neugeberger, F. A., Blaschowski, H. P., & Ganzler, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1332–1335.

Moss, M. L., & Frey, P. A. (1987) *J. Biol. Chem. 262*, 14859–14861.

Moss, M. L., & Frey, P. A. (1990) *J. Biol. Chem. 265*, 18112–18115.

Petrovich, R. M., Ruzicka, F. J., Reed, G. H., & Frey, P. A. (1991) *J. Biol. Chem. 266*, 7656–7660.

Stadtman, T. C. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 38*, 413–448.