Lysine 2,3-Aminomutase: Rapid Mix−Freeze−Quench Electron Paramagnetic Resonance Studies Establishing the Kinetic Competence of a Substrate-Based Radical Intermediate†

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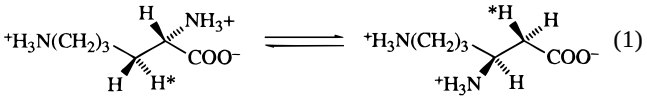
ABSTRACT: Lysine 2,3-aminomutase from *Clostridia* catalyzes the interconversion of L-lysine and L-β-lysine. The enzyme contains iron−sulfur clusters and is activated by pyridoxal 5′-phosphate and *S*-adenosylmethionine, all of which participate in catalysis. Current spectroscopic evidence implicates two substrate-based organic radicals as intermediates in the mechanism. One of these species, the radical N^3-(5′-phosphopyridoxylidene)-β-lysin-2-yl (**3**), appears in the steady state of the reaction of lysine and has been definitively characterized by EPR and ESEEM spectroscopy. The 2-deuterio form of this radical, **3**-*2-d*, which is generated in the reaction of L-[2-^{2}H]lysine, can be distinguished by line shape analysis from **3**. The rate at which the signal for **3**-*2-d* is transformed into that for **3** has been measured by rapid mix−freeze quench kinetic analysis. The rate constant for this process is 24 ± 8 s^{-1} at 21 °C. This is the rate constant for the turnover of radical **3** and is indistinguishable from the turnover number of lysine 2,3-aminomutase. Therefore, radical **3** is kinetically competent as an intermediate in the reaction of lysine 2,3-aminomutase.

Lysine 2,3-aminomutase catalyzes the reversible isomerization of L-lysine into L-β-lysine, a reaction in which the hydrogen in the 3-*pro-R* position of lysine is transferred to the 2-*pro-R* position of β-lysine and the 2-amino group of lysine migrates to carbon-3 of β-lysine (Chirpich et al., 1970; Aberhart et al., 1983). The migrating hydrogen does not exchange with solvent hydrogen, and the stereochemistry of its migration is illustrated in eq 1, in which the transferred hydrogen is marked with an asterisk. The enzyme from

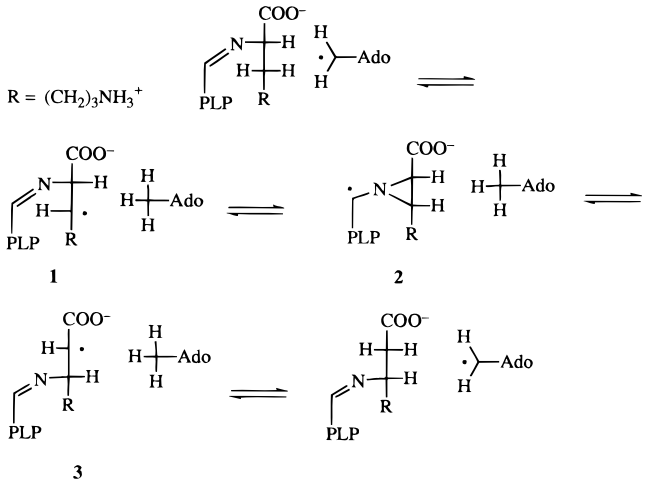


(1)

Clostridium SB4 contains iron, requires PLP,[1] and is activated by *S*-adenosylmethionine (Chirpich et al., 1970). The 5′-deoxyadenosyl group of *S*-adenosylmethionine mediates the hydrogen transfer process (Moss & Frey, 1987; Baraniak et al., 1989), and iron associated with the enzyme exists in the form of an iron−sulfur center (Petrovich et al., 1992).

The chemical mechanism is unique among isomerization reactions. Most enzymatic isomerizations of the same type require adenosylcobalamin as the coenzyme, and reversible homolytic cleavage of its cobalt−carbon bond within enzymatic sites is thought to generate the 5′-deoxyadenosyl radical (5′-deoxyadenosin-5′-yl), which initiates radical rearrangements. Lysine 2,3-aminomutase appears to use the iron−sulfur center and *S*-adenosylmethionine to generate the 5′-deoxyadenosyl radical, which is postulated to initiate lysine isomerization by the mechanism shown in Scheme 1 (Moss & Frey, 1987; Frey, 1990; Frey & Reed, 1992).

Scheme 1



Extensive spectroscopic evidence implicates radical **3** in Scheme 1 as an intermediate (Ballinger et al., 1992a,b, 1995). Recent spectroscopic evidence on the reaction of the alternative substrate 4-thialysine implicates radical **1** as an intermediate. Radicals **1** and **3** appear in the steady states of the enzymatic isomerizations of substrates, and the concentration of radical **3** declines as substrate equilibration proceeds. However, neither radical has heretofore been established as kinetically competent to serve as an intermediate. In this paper, we prove that radical **3** is kinetically competent in the transformation of lysine into β-lysine.

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[1] Abbreviations: EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; SAM, *S*-adenosylmethionine; PLP, pyridoxal 5′-phosphate; CM, carboxymethyl.

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EXPERIMENTAL PROCEDURES

Chemicals and Substrates. *S*-Adenosylmethionine was purchased from Sigma and purified by adsorption to a column of CM-cellulose in the Na^+ form at pH 5.0. *S*-Adenosylmethionine was eluted with 40 mM HCl. Pooled fractions, chosen on the basis of A_{260}, were adjusted to pH 5.0 by addition of Q-Sepharose in the hydroxide form, filtered to remove the Sepharose beads, and concentrated by rotary evaporation *in vacuo* to 85 mM. The solution was frozen in liquid nitrogen and stored at −70 °C. Lysine hydrochloride was converted into its sulfate salt. DL-[2-^{2}H]-Lysine was synthesized by the method of Battersby et al. (1982), modified as described elsewhere (Ballinger et al., 1992b). Lysine 2,3-aminomutase uses only the L-isomer and is not inhibited significantly by the D-isomer under the conditions of the present experiments. Buffers and other chemicals were purchased in the highest available purity and used as supplied.

Enzyme Purification and Assay. Lysine 2,3-aminomutase was activated and assayed according to the procedure described by Chirpich et al. (1970) as amended by Ballinger et al. (1992a). The enzyme was purified by the procedure of Moss and Frey (1990) as amended by Petrovich et al. (1991). All procedures were carried out within a Coy anaerobic chamber except for preparative centrifugations, in which centrifuge bottles filled and sealed in the anaerobic chamber were removed for centrifugation and later returned to the chamber before being opened. The purified enzyme exhibited a specific activity of approximately 30 IU/mg of protein in the standard assay.

Rapid Mix−Quench EPR Kinetics. Transient phase kinetic studies were carried out in a rapid mix−freeze quench apparatus built by the Update Instrument Co. (Madison, WI) and equipped with an Update Model 745 Syringe-Ram Controller. Syringes and timing tubes were held in the Coy anaerobic chamber for several days in advance of each session to remove dioxygen. Early in the day for a kinetic run, the rapid mix apparatus was assembled and cooled with liquid nitrogen. Lysine 2,3-aminomutase was then placed in the anaerobic chamber and incubated with reduction buffer for 4 h at 37 °C, during which the isopentane bath, collection funnels, and anaerobic pot for use in freeze quenching were slowly cooled to −140 °C. Solutions for loading syringes were prepared in the anaerobic chamber. The enzyme was assayed prior to loading the syringes to verify its activity. All syringe solutions were prepared at pH 8.0. Syringe 1 contained the reduced enzyme with 10 mM dithionite. Syringe 2 contained the activation mixture consisting of 40 mM Tris-SO_4, 160 mM DL-[2-^{2}H]lysine·$NaSO_4$, 4 mM *S*-adenosylmethionine, and 10 mM dithionite. Syringe 3 contained the substrate solution consisting of 40 mM Tris-SO_4, 450 mM L-lysine·$NaSO_4$, and 10 mM dithionite. The solutions were drawn into the syringes immediately after preparation, and the loaded syringes were removed from the anaerobic chamber and bolted onto the computer-controlled ram device. The values for the given time point were entered into the ram control program, and the sample was shot. The funnel and EPR tube were transferred to the isopentane bath and the frozen crystals of sample allowed to settle while another sample was collected. The crystals from each kinetic run were packed into the bottom of an EPR tube at −140 °C by gently pushing them down with a thin metal rod. After packing, the isopentane in the EPR tube and collection funnel was removed, the funnel was replaced with a small extending tube, and the sample was plunged into liquid nitrogen and stored at 77 K until data acquisition.

EPR spectra were recorded at X-band on a Varian E3 spectrometer interfaced with an IBM AT-type computer. Samples were contained in a standard liquid N_2 immersion dewar. Frequency was calibrated with a Hewlett-Packard frequency meter. Spectra were acquired as an average of four 200 G scans. To facilitate calculation of difference spectra and integrals, baseline corrections were applied to the spectra to adjust the slope to zero. The observed spectrum at time *t*, $[S'_{obs(t)}]$ is the sum of the spectra for the protio and deuterio components, $S'_{obs(t)} = aS'\mathbf{3} + bS'\mathbf{3}$-2-*d*, where *a* and *b* are, respectively, the fraction of total signal due to the protio and deuterio components. A fraction, *f*, of a reference spectrum, $S'\mathbf{3}_{ref}$, was subtracted from $S'_{obs(t)}$ to give a difference spectrum, $S'_{diff(t)}$, that corresponded to the line shape of a spectrum of pure **3**-*2*-*d*. The fraction of the protio component, *a*, at time *t* was obtained from the double integrals of the spectra according to eq 2.

$$a = \frac{[\mathbf{3}]}{[\mathbf{3}] + [\mathbf{3}\text{-}2\text{-}d]} = \frac{1}{1 + \dfrac{\int\int S'_{diff(t)}}{f\int\int S'\mathbf{3}_{ref}}} \qquad (2)$$

RESULTS AND DISCUSSION

*Kinetic Competence of Radical **3**.* Proof of kinetic competence for a putative intermediate in an enzymatic reaction ideally entails the measurement of two rates, that at which the species is produced from a substrate and that at which it reacts further to form the product or another intermediate. Rapid mix−quench methods are often used to monitor the formation and further reactions of intermediates. In the case of lysine 2,3-aminomutase, the reactions of radical **3** can in principle be monitored by rapid mix−freeze quench EPR; however, the properties of the enzyme make it impossible to test the kinetic competence of radical intermediates by the conventional application of this method. The purified enzyme must be activated by *S*-adenosylmethionine, but its activity is labile in the absence of lysine so that maximal activation is facilitated by the presence of both the substrate and the coenzyme. Moreover, activation by *S*-adenosylmethionine requires about 5 s and so is much slower than enzymatic turnover. Therefore, the rate at which **3** appears in conventional rapid mix−freeze quench EPR experiments tracks the activation by *S*-adenosylmethionine and not the rate at which **3** is produced by the activated enzyme (Ballinger, 1993).

Another way to evaluate kinetic competence is to determine whether the putative intermediate turns over as fast as overall enzymatic turnover. A species that turns over slower than enzymatic turnover cannot be an intermediate, but all intermediates must turn over at least as fast as enzymatic turnover. The turnover rate for **3** can be measured in a rapid mix−freeze quench apparatus that is programmed to carry out the operations diagrammed in Scheme 2. Enzyme in one syringe is mixed with [2-^{2}H]lysine and *S*-adenosylmethionine from a second syringe (pulse), and the mixture is held in the flow train for 5 s to generate the radical **3**-*2*-*d*.

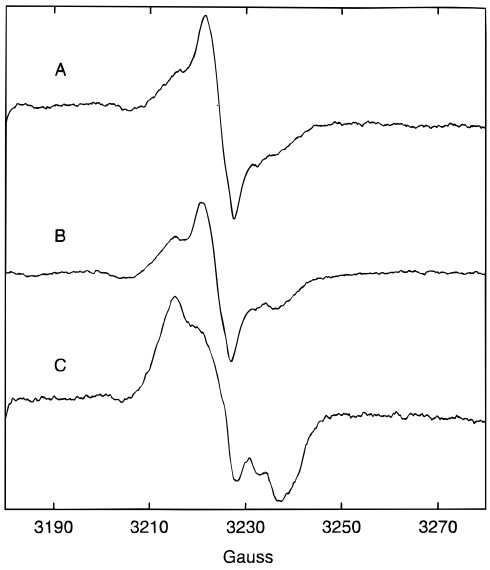
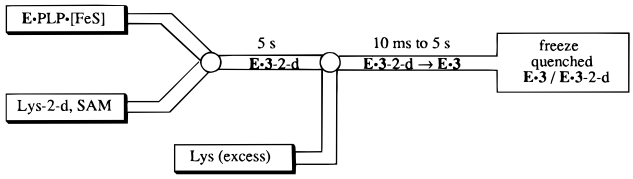


FIGURE 1: EPR spectra showing the transformation of radical **3**-*2-d* into **3**. Reaction conditions are described in Experimental Procedures. Spectra A and B were obtained on samples quenched after 13.6 and 53 ms, respectively. The broadening at 53 ms relative to 13.6 ms corresponds to the increase in the ratio **3**/**3**-*2-d* over this time period. Spectrum C was obtained on a sample quenched after 5 s and represents the ratio of **3** and **3**-*2-d* resulting from the dilution of 80 mM DL-[2-^{2}H]lysine (40 mM L-[2-^{2}H]lysine) with 225 mM L-lysine.

Scheme 2



Sufficient [2-^{2}H]lysine is used to ensure that the reaction is in its steady state at 5 s. The flow is repeated with a third syringe containing a large excess of unlabeled lysine (chase) so that in the last segment of the flow train radical **3**-*2-d* is transformed into radical **3** by enzymatic turnover. The reaction is quenched by spraying into liquid isopentane as a function of time. Radicals **3**-*2-d* and **3** in the quenched samples can be distinguished by their EPR spectra because of the smaller value of the deuterium hyperfine coupling constant in the spectrum of **3**-*2-d* (Ballinger et al., 1992a,b).

Representative EPR data are shown in Figure 1 to illustrate the spectral changes that take place following the chase. The spectrum of Figure 1A is that obtained for a sample quenched 13.6 ms into the second flow stage so that mainly radical **3**-*2-d* is observed with a small content of **3**. The spectrum in Figure 1B is that from an experiment in which the reaction was quenched at 53 ms. A substantial broadening of the EPR signal relative to that in Figure 1A demonstrates the further transformation of the spectrum of **3**-*2-d* into that of **3**. Analysis of the spectrum showed that the ratio **3**/**3**-*2-d* in this experiment was 2.7 at the time of quenching. Figure 1C is the spectrum 5 s into the second flow, when it corresponds to the steady state in the reaction of 40 mM [^{2}H]lysine and 150 mM lysine. This represents the end point of the enzymatic turnover of radical **3**-*2-d* into **3**.

Repetitions of these experiments, in which the quenching times varied, allowed the ratio **3**/**3**-*2-d* to be calculated as a function of time between 10 ms and 5 s in the second flow stage. The resulting data, illustrating the smooth first-order transformation of **3**-*2-d* into **3** over the course of about 200 ms, are displayed in Figure 2. The first-order rate constant for the turnover of the radical, calculated from the data in Figure 2, is 24 ± 8 s^{-1}.

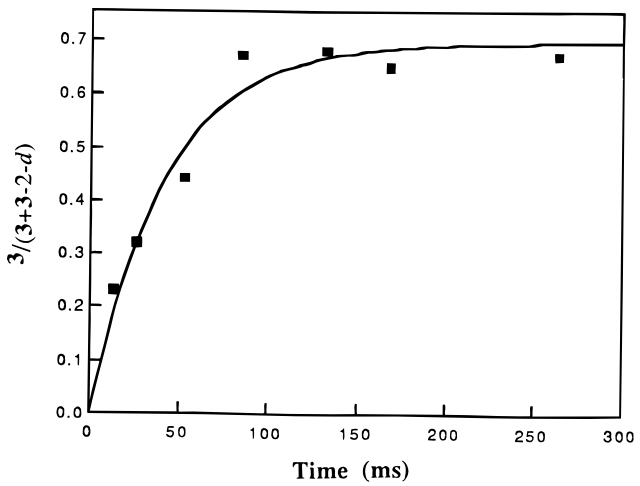


FIGURE 2: Time course for the development of radical **3** from **3**-*2-d* in the active site of lysine 2,3-aminomutase. The growing fraction of **3** in the radical mixture at 21 °C is plotted as a function of quenching time for a series of experiments similar to those described in Figure 1. The data were fitted to a first-order equation by use of the program Ultrafit (Biosoft, Ferguson, MO). The rate constant is 24 ± 8 s^{-1}.

The best preparations of lysine 2,3-aminomutase exhibit specific activities of about 40 IU/mg, measured at 37 °C and at substrate saturation. Lysine 2,3-aminomutase is reported to be a hexamer of identical subunits (Chirpich et al., 1970; Song & Frey, 1992). Assuming six active sites per molecule of enzyme and that the enzyme is homogeneous, the turnover number should be 32 s^{-1} at 37 °C. The data in Figure 2 were obtained at 21 °C so that the turnover number at 37 °C does not apply and must be corrected to 21 °C. Assay of a sample of the enzyme in triplicate at both temperatures showed it to be 43% as active at 21 °C as at 37 °C so that the turnover number corrected to 21 °C is 14 s^{-1}. To the extent that the enzyme contained impurities or nonfunctional active sites, owing to imperfect activation, this estimate for the turnover number will be lower than the true value. The value of 24 s^{-1} for the turnover of radical **3** is somewhat faster than the estimated enzymatic turnover; therefore, radical **3** is kinetically competent as an intermediate.[2]

Radical Intermediates in the Action of Lysine 2,3-Aminomutase. Scheme 1 shows four organic radicals as intermediates in the catalytic cycle of lysine 2,3-aminomutase: the

[2] The true enzymatic turnover number at 21 °C and pH 8.0 is likely to be 24 ± 8 s^{-1} as determined from the data in Figure 1, and the value of 14 s^{-1} estimated from activity measurements could be low if there is incomplete activation of sites. Spin counts on the intermediate radical **3** indicate that this species corresponds to at least 10% of the subunits in the steady state at saturating substrate (Ballinger et al., 1995). The inhibitor *trans*-4-dehydrolysine reacts with the activated enzyme to produce an allylic radical corresponding to intermediate **1** in Scheme 1 (K. Lieder, W. Wu, G. H. Reed, and P. A. Frey, to be published). Spin counts on this radical indicate that approximately half the subunits carry this radical in the inhibited enzyme. Therefore, at least half of the subunits are functional in our enzyme preparations. This means that the kinetic value for the turnover number at 21 °C cannot be larger than 28 s^{-1}, which is within error of the turnover number for the radical **3**.

5′-deoxyadenosyl radical and substrate-based radicals **1**−**3**. In the steady state of the reaction of lysine, radical **3** was initially observed by its EPR spectrum; it appeared within 30 s of mixing lysine and *S*-adenosylmethionine with the enzyme (Ballinger et al., 1992a). This radical signal was at maximal intensity within seconds of mixing the substrate with the active enzyme, and it declined to an equilibrium intensity in parallel with the approach to overall equilibrium between lysine and β-lysine, which favors β-lysine. This initial observation was consistent with **3** being an intermediate in the overall reaction; however, it did not establish kinetic competence. The structure of radical **3** was subsequently established spectroscopically in EPR studies that proved its conformation to be consistent with the stereochemistry of hydrogen transfer (Ballinger et al., 1992b). Strong evidence that the radical **3** includes PLP in aldimine linkage has recently been provided by the results of deuterium ESEEM spectroscopy (Ballinger et al., 1995). The present proof that **3** is kinetically competent to be an intermediate rounds out the evidence for its role in the mechanism.

The transformation of [3,3-$^2H_2$]lysine into β-[2,3-$^2H_2$]-lysine by lysine 2,3-aminomutase proceeds with a kinetic isotope effect of 2.9 (Aberhart, 1988), suggesting that hydrogen transfer is rate-limiting or partially rate-limiting. In the mechanism of Scheme 1, hydrogen transfer takes place between 5′-deoxyadenosine and two substrate radicals, intermediates **1** and **3**. Both of these steps can be expected to exhibit a deuterium kinetic isotope after the first few turnovers, which lead to the formation of 5′-[5′-$^2H_3$]-deoxyadenosine as an intermediate. Either of these steps may be rate-limiting or partially rate-limiting; the identification of the rate-limiting step must await a more complete kinetic analysis of the overall reaction.

That Scheme 1 is a reasonable description of the mechanism has recently been buttressed by the observation of the 4-thia analog to radical **1** in the reaction of the alternative substrate 4-thialysine (Wu et al., 1995). In the reaction of lysine, **3** is the most stable radical in the mechanism because the unpaired electron is delocalized into the carboxyl group and there are no destabilizing structural features. In the reaction of 4-thialysine, sulfur in place of C-4 stabilizes the 4-thia analog of **1** enough to make it the most stable radical in the mechanism so that it is the only species observed by EPR. Species **2** of Scheme 1 has not been observed in EPR spectra. It should be electronically stabilized through delocalization of the unpaired electron into the pyridine ring of PLP; however, it is destabilized by bond angle strain in the aziridine ring. In the balance, it is apparently not stable enough to be observed in EPR experiments, at least not in the presence of the more stable radical **3** or the 4-thia analog of **1**. The 5′-deoxyadenosyl radical, which lacks electronic stabilizing features in its structure, is also not observed in EPR experiments. Despite the absence of evidence for radical **2** and the 5′-deoxyadenosyl radical, the mechanism is supported by the characterization of radicals **3** and the 4-thia analog of **1** in the steady state. Proof of the kinetic competence of **3**, as set forth in this paper, substantially consolidates the evidence for Scheme 1 as the isomerization mechanism.

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