Lysine 2,3-Aminomutase and *trans*-4,5-Dehydrolysine: Characterization of an Allylic Analogue of a Substrate-Based Radical in the Catalytic Mechanism†

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ABSTRACT: An analogue of lysine, *trans*-4,5-dehydro-L-lysine (*trans*-4,5-dehydrolysine), is a potent inhibitor of lysine 2,3-aminomutase from *Clostridium subterminale SB4* that competes with L-lysine for binding to the active site. Inclusion of *trans*-4,5-dehydrolysine with activated enzyme and the coenzymes pyridoxal-5′-phosphate and *S*-adenosylmethionine, followed by freezing at 77 K, produces an intense signal in the electron paramagnetic resonance (EPR) spectrum at *g* 2.0, which is characteristic of an organic radical. A series of deuterated and ^{15}N-labeled samples of *trans*-4,5-dehydrolysine were synthesized and used to generate the EPR signal. Substitution of deuterium for hydrogen at C2, C3, C4, C5, and C6 of *trans*-4,5-dehydrolysine led to significant simplifications and narrowing of the EPR signal, showing that the unpaired electron was located on the carbon skeleton of 4,5-*trans*-4,5-dehydrolysine. The hyperfine splitting pattern is simplified by use of 4,5-dehydro[3,3-$^2H_2$]lysine or 4,5-dehydro[4,5-$^2H_2$]lysine, and it is dramatically simplified with 4,5-dehydro-[3,3,4,5,6,6-$^2H_6$]lysine. Spectral simulations show that the EPR signal arises from the allylic radical resulting from the abstraction of a hydrogen atom from C3 of *trans*-4,5-dehydrolysine. This radical is an allylic analogue of the substrate-related radical in the rearrangement mechanism postulated for this enzyme. The rate constant for formation of the 4,5-dehydrolysyl radical (2 min^{-1}) matches that for the decrease in the concentraion of $[4Fe\text{-}4S]^+$, showing that the two processes are coupled. The cleavage of *S*-adenosylmethionine to 5′-deoxyadenosine and methionine takes place with a rate constant of approximately 5 min^{-1}. These kinetic correlations support the hypothesis that radical formation results from a reversible reaction between $[4Fe\text{-}4S]^+$ and *S*-adenosylmethionine at the active site to form $[4Fe\text{-}4S]^{2+}$, the 5′-deoxyadenosyl radical, and methionine as intermediates.

Lysine 2,3-aminomutase from *Clostridium subterminale SB4* catalyzes the interconversion of L-lysine and L-β-lysine (eq 1). The enzyme is a hexamer of identical subunits (47 kDa) with an overall molecular mass of 280 kDa (*1*, *2*). The protein contains [4Fe-4S] centers and is activated by PLP[1] and AdoMet. The transfer of hydrogen between C2 and C3 of lysine and β-lysine, respectively, proceeds without exchange with solvent protons (*1*).

(1)

The chemical mechanism in Figure 1 involving the 5′-deoxyadenosyl radical and lysyl radical intermediates ***a***, ***b***, and ***c*** has been proposed (*3*−*5*). Spectroscopic evidence

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[1] Abbreviations: *trans*-4,5-dehydrolysine, (*E*)-4,5-dehydro-DL-lysine; 2,3-aminomutase, lysine 2,3-aminomutase; PLP, pyridoxal-5′-phosphate; lysine, L-lysine; β-lysine, β-L-lysine; ATP, adenosine-5-triphosphate; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; Tris, tris-(hydroxyethyl)aminomethane; Epps, *N*-[2-hydroxyethyl]-piperazine-*N*′-[3-propanesulfonic acid]; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; DMF, dimethylformamide; PITC, phenylisothiocyanate; DIBAL, diisobutylaluminum; CM-cellulose, carboxymethyl cellulose; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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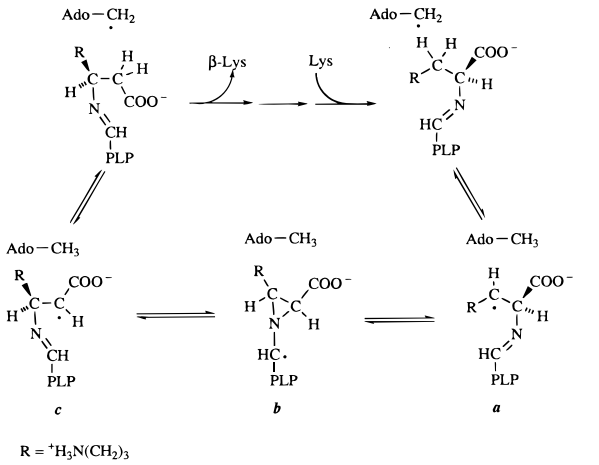
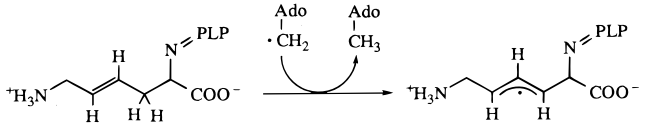


FIGURE 1: Mechanism of substrate radical initiation and isomerization in the reaction of lysine 2,3-aminomutase.

indicates that a reaction between AdoMet and the reduced iron−sulfur center ($[4Fe\text{-}4S]^{+}$) leads to the reversible formation of the 5′-deoxyadenosyl radical and the initiation of the rearrangement reaction, the mechanism of which is illustrated schematically in Figure 1 (*6*). The 5′-deoxyadenosyl radical (Ado-CH_2• in Figure 1) is postulated to abstract a hydrogen atom from C3 of lysine, which is bound to PLP as its external aldimine. The initiation step forms 5′-deoxyadenosine and the substrate-related radical ***a***, which undergoes rearrangement to an azacyclopropylcarbinyl radical ***b***, a quasi-symmetric species that rearranges further to the product-related radical ***c*** in Figure 1. Abstraction of a hydrogen from the methyl group of 5′-deoxyadenosine by radical ***c*** regenerates the 5′-deoxyadenosyl radical and produces β-lysine in the form of its external aldimine with PLP. The mechanism is novel in that, although the overall aminomutation is analogous to adenosylcobalamin-dependent reactions, no vitamin B_{12} coenzyme is involved, and AdoMet supplies the 5′-deoxyadenosyl moiety that mediates hydrogen transfer from C3 to C2 in the transformation of lysine into β-lysine. The role of PLP in facilitating the radical rearrangement is also a departure from its normal role of stabilizing amino acid carbanions.

The product radical ***c*** from the reaction of the enzyme with lysine (*7*, *8*) and a substrate radical analogue of ***a*** in the reaction with the alternative substrate 4-thialysine (*9*) have been observed and characterized spectroscopically. The structure of the product radical ***c*** in Figure 1 is known, as determined by EPR and ESEEM spectroscopies (*8*, *10*), and it is kinetically competent as an intermediate (*11*). The observation of the substrate radical ***a*** in Figure 1 by EPR spectroscopy depended on the use of a structural analogue of lysine as a substrate. 4-Thialysine was found to be an alternative substrate and led to the 4-thia analogue of radical ***a*** as the dominant paramagnetic species in the steady-state of the reaction (*9*). The stability of the 4-thia analogue of radical ***a*** was attributed to electronic stabilization of the unpaired electron on C3 by the adjacent sulfur atom (*9*).

Another analogue of lysine is *trans*-4,5-dehydrolysine. The 4,5-olefinic bond is in a position to stabilize an unpaired electron at C3. If *trans*-4,5-dehydrolysine is recognized as a substrate that undergoes the initial hydrogen abstraction from C3, an allylic radical should result as follows:



The allylic radical is likely to be stable enough to accumulate to an observable concentration in an enzymatic experiment. In this paper, we show that *trans*-4,5-dehydrolysine is a potent inhibitor that is converted into the allylic radical in the active site. The stability of this radical has allowed its equilibrium fractional concentration to be correlated with the fractional presence of the two putative functional states of the iron−sulfur center.

EXPERIMENTAL PROCEDURES

Chemicals. L-lysine, L-leucine, L-methionine, inorganic pyrophosphatase (baker's yeast), AdoHcy, ATP, 5′-deoxyadenosine, CM-cellulose, Epps, and Hepes were purchased from Sigma Chemicals. AdoMet (*p*-toluenesulfonate salt) was also purchased from Sigma and purified by CM-cellulose chromatography, with elution by 20 mM H_2SO_4. PITC was purchased from Pierce. Q-Sepharose and phenyl sepharose resins were obtained from Pharmacia, and sodium dithionite was obtained from Aldrich. [U-^{14}C]Lysine was purchased from New England Nuclear, [*adenosine*-8-^{14}C]ATP (SA 47 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc., and L-[*methyl*-^{14}C]methionine (SA 50 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. Anhydrous solvents for chemical synthesis were purchased from Aldrich Chemical Co. and used as supplied. Diisobutylaluminum deuteride (DIBAL-D, ~50% D), lithium aluminum deuteride ($LiAlD_4$, 98% D), deuterium chloride (DCl) solution in D_2O (99.5% D), D_2O (99.9% D), acetic acid-*d* (98% D) were also obtained from Aldrich Chemical Co. 1,3-Butadiene-d_6 (98% D) and potassium [^{15}N]phthalimide were purchased from Cambridge Isotope Laboratories, Inc. All other chemicals and buffers were obtained from vendors and used as supplied unless otherwise stated.

Assays. The standard assay of 2,3-aminomutase used during purification and for measuring its specific activity has been described (*8*). *trans*-4,5-Dehydrolysine was assayed by HPLC after derivatization with PITC (*12*). To examine the possible substrate reactivity of *trans*-4,5-dehydrolysine, 2,3-aminomutase was reductively incubated by the usual procedure to prepare it for a standard assay (*8*). An aliquot (50 μL) of the enzyme was added to 65 μL of an assay mixture at 37 °C consisting of 0.26 M Epps buffer at pH 8.0, 2.3 mM $Na_2S_2O_3$, 1.2 mM AdoMet, and 20 mM *trans*-4,5-dehydrolysine, combined in that order. After incubation for 30 min, the reaction was quenched with 25 μL of 2 M $HClO_4$. The precipitated protein was removed by centrifugation, and an aliquot of the supernatant fluid was derivatized for HPLC analysis.

UV−vis spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer. HPLC was carried out on an integrated system from Waters consisting of 501 and 510 pumps, a 484 tunable absorbance detector, and an automatic gradient controller. An integrator from Spectrum Inc. determined relative areas of peaks. Radiochemical analysis was carried out by liquid scintillation counting.

trans-4,5-Dehydrolysine·2HCl was passed over a column of Dowex 1-AG column in the HSO_4^- form to remove the Cl^-. Ninhydrin positive fractions were pooled and concentrated in vacuo. The concentration of *trans*-4,6-dehydrolysine was determined colorimetrically with ninhydrin, employing L-lysine as a standard.

Enzymes. Lysine 2,3-aminomutase was purified from *Clostridium subterminale SB4* as described (*7*). The concentration of the purified enzyme ranged from 22 to 37 mg mL^{-1}, and the specific activities ranged from 35 to 40 U mg^{-1}. All manipulations of 2,3-aminomutase were carried out in an anaerobic chamber equipped with a model 10 Oxygen-Hydrogen Analyzer (Coy Laboratory Products, Inc.). Palladium catalysts were used to maintain the oxygen tension below 1 ppm, the limit of detection.

AdoMet synthetase was purified from *Escherichia coli* overproducing strain DM22pk8 as previously described (*13*, *14*), except that the final step, chromatography on aminohexyl-sepharose (AH4B), was omitted. The enzyme was judged to be at least 85% pure by SDS−PAGE and exhibited a specific activity of 1 U mg^{-1}.

Synthesis of Radiolabeled Compounds. [*adenosyl*-8-^{14}C]-AdoMet and [*methyl*-^{14}C]AdoMet were synthesized enzymatically using AdoMet synthetase. The reactions contained the following in a final volume of 10 mL: 50 mM Hepes at pH 8.0, 50 mM KCl, 20 mM $MgCl_2$, 100 U of inorganic pyrophosphatase, and 5 U of AdoMet synthetase. For the synthesis of [*adenosyl*-8-^{14}C]AdoMet, the reactions also contained [*adenosyl*-8-^{14}C]ATP and L-methionine at 1 and 5 mM, respectively; in the case of [*methyl*-^{14}C]AdoMet, the reaction contained 5 mM ATP and 2.2 mM radiolabeled methionine. The solutions were incubated at ambient temperature (21 ± 2 °C) for 40 min, diluted into 200 mL of 1 mM Na-acetate (pH 5.0), and loaded onto a CM-cellulose column (2.5 × 7 cm) equilibrated in the same buffer. After washing the column with the 1 mM Na-acetate buffer (200 mL), AdoMet was eluted with 20 mM H_2SO_4. A_{260}-positive fractions were pooled, titrated to pH 5.0 with Q-sepharose (OH^- form), and concentrated by flash evaporation to 2−3 mM. The final specific activities of [*adenosyl*-8-^{14}C]AdoMet and [*methyl*-^{14}C]AdoMet were 3.4×10^6 and 2.1×10^6 cpm μmol^{-1}, respectively.

Synthesis of Deuterium-Labeled Compounds. All reactions were carried out under dry nitrogen. Analytical TLC was performed on 0.2 mm silica 60 coated plastic plates with F-254 indicator. Flash chromatography was performed on Merck 230−400 mesh silica gel as described by Still et al. (*15*). ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or D_2O. Chemical shifts are reported in parts per million (ppm) and coupling constants are reported in hertz (Hz). ^{1}H NMR spectra were referenced to tetramethylsilane and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (sodium salt) at 0.00 ppm in $CDCl_3$ and D_2O, respectively. ^{13}C NMR spectra were referenced to the solvent peak at 77.0 ppm in $CDCl_3$.

For the synthesis of labeled *trans*-4,5-dehydrolysine, only the procedures leading to labeled ethyl (*E*)-2-carbethoxy-2,6-diphthalimido-4-hexenoate (**8**) are described below, because the hydrolysis of this precursor to labeled (*E*)-4,5-dehydro-DL-lysine·2HCl (**1**) follows the procedure for unlabeled material. ^{1}H NMR spectra of labeled amino acids are consistent with deuterium incorporation at the desired positions.

Ethyl (E)-2-carbethoxy-2,6-diphthalimido-4-hexenoate, **8.** Diethyl phthalimidomalonate (90 mg, 0.30 mmol) was mixed with excess sodium hydride (60% dispersion in mineral oil) in anhydrous toluene, and the mixture was stirred at 80 °C for 90 min. Upon cooling the solution to room temperature, bromide **7** (70 mg, 0.25 mmol) was added, and the mixture was stirred for 5 min. The solvent was removed at reduced pressure, and the solid residue was heated at 155−160 °C for 3 h. Upon cooling the mixture, the residue was triturated with chloroform, filtered, and concentrated to give a yellow solid. The product was purified by flash chromatography (5% ethyl acetate-methylene chloride or 20−50% ethyl acetate−hexane) to give **8** (110 mg, 84%) as a white solid: ^{1}H NMR δ 7.69−7.67 (m, 6H, aromatic), 7.59−7.57 (m, 2H, aromatic), 5.96 (p, J = 15.3, J = 7.5, 1H, vinylic), 5.50 (p, J = 15.3, J = 7.5, 1H, vinylic), 4.35−4.22 (m, 4H, OCH_2), 4.04 (d, J = 7.5, 2H, NCH_2), 3.17 (d, J = 7.5, 2H, CH_2), 1.26 (t, J = 6.7, 6H, CH_3); ^{13}C NMR δ 167.54, 167.08, 166.01, 134.19, 133.93, 132.18, 131.56, 129.08, 128.88, 123.56, 123.32, 67.60, 63.00, 39.23, 36.47, 14.05.

(*E*)-4,5-Dehydro-DL-lysine dihydrochloride [(±)-(*E*)-2,6-diamino-4-hexenoic acid·HCl], **1**. Imidoester **8** was suspended in concentrated HCl or a mixture of 6 N aqueous HCl and acetic acid (3 to 5:1 by volume) and refluxed for 20 h. Upon cooling, the solution was evaporated to dryness under reduced pressure. A small volume of concentrated HCl was added to the residue and removed under reduced pressure. The process was repeated with concentrated HCl and with ethanol. The resulting residue was triturated with ethanol and ether and filtered to give **1** as a white solid. The ninhydrin reaction produced a yellow spot that slowly turned purple (*16*). ^{1}H NMR δ 5.90−5.80 (m, 2H, vinylic), 4.11 (t, J = 6.0, 1H, α-H), 3.62 (d, J = 5.2, 2H, NCH_2), 2.79−2.71 (m, 2H, CH_2).

Ethyl (*E*)-2-Carbethoxy-2,6-diphthalimido-4-hexenoate-[4,5-$^2H_2$]. 2-Butyne-1,4-diol (2.9 g, 33.7 mmol) in anhydrous tetrahydrofuran (15 mL) was added dropwise with stirring to a slurry of lithium aluminum deuteride (1.9 g, 45.2 mmol) in anhydrous ether (80 mL), and the mixture was refluxed for 4 h. Upon cooling the mixture, a minimum amount of cold D_2O was added dropwise to the reaction until the gray slurry just turned white (3−4 mL). The mixture was filtered, the solid was washed with ether, and the combined organic solution was dried over $MgSO_4$ and concentrated to a colorless oil, which solidified upon refrigeration to give (*E*)-butene-1,4-diol-[2,3-$^2H_2$] as a white solid. The IR spectrum showed loss of C≡C in the product, which was used without further purification.

The diol (190 mg, 2.1 mmol) and carbon tetrabromide (1.54 g, 4.6 mmol) were dissolved together in anhydrous acetonitrile (12 mL), triphenylphosphine (1.22 g, 4.6 mmol) was added in portions at −40 °C, and the mixture was brought slowly to room temperature and stirred for 3 h. The solvent was removed under reduced pressure, and hexane was added to give a suspension, which was filtered and washed thoroughly with hexane. The filtrate and wash were combined and concentrated to a colorless oil, which soon solidified to give (*E*)-1,4-dibromo-2-butene-[2,3-$^2H_2$], which was used directly in the next step. ^{1}H NMR δ 3.94 (s).

The product was converted to (*E*)-1-bromo-4-phthalimido-2-butene-[2,3-$^2H_2$] (not isolated) by the procedure of Birkofer and Hempel (*17*) as a white solid: ^{1}H NMR δ 7.87−7.85

(m, 2H, aromatic), 7.74−7.73 (m, 2H, aromatic), 4.31 (s, 2H, NCH_2), 3.91 (s, 2H, CH_2Br).

The bromide was used directly in the reaction with diethyl 2-phthalimidomalonate as described for the preparation of **8** to give ethyl (*E*)-2-carbethoxy-2,6-diphthalimido-4-hexenoate-[4,5-$^2H_2$] as a white solid. TLC indicated that the product was a single compound that comigrated with **8**. The ^{1}H NMR spectrum verified the absence of hydrogen at C-4 and C-5. ^{1}H NMR δ 7.69−7.67 (m, 6H, aromatic), 7.59−7.57 (m, 2H, aromatic), 4.34−4.24 (m, 4H, OCH_2), 4.03 (s, 2H, NCH_2), 3.16 (s, 2H, CH_2), 1.26 (t, J = 7.1, 6H, CH_3).

Ethyl (*E*)-2-Carbethoxy-2,6-diphthalimido-4-hexenoate-[3,3-$^2H_2$]. To a solution of methyl 4-bromocrotonate (250 mg, 164 mL, 1.4 mmol) in anhydrous toluene (5 mL) was added dropwise a 0.5 M solution of DIBAL-D (∼50% D) in toluene (7.0 mL, 3.5 mmol) at −78 °C, and the solution was stirred for 30 min. Aqueous acetic acid (50%, 0.4 mL, 3.5 mmol) was added, the mixture was brought to room temperature and filtered through a Celite pad, and the pad was washed with acetone (5 × 5 mL). The combined filtrates were concentrated to give 4-bromo-2-butene-1-ol-[1,1-$^2H_2$] (not isolated) as a yellow oil. A small portion of the product was treated with excess potassium phthalimide in anhydrous DMF according to the procedure of Sheehan and Bolhofer (*18*) and gave the crude product as a yellow solid. Flash chromatography (20% ethyl acetate−hexane) afforded 4-phthalimido-2-butene-1-ol-[1,1-$^2H_2$] as an off-white solid. The ^{1}H NMR spectrum indicated ∼58% deuterium incorporation. ^{1}H NMR δ 7.87−7.84 (m, 2H, aromatic), 7.75−7.71 (m, 2H, aromatic), 5.91−5.86 (m, 1H, vinylic), 5.83−5.76 (m, 1H, vinylic), 4.31 (d, J = 8.0, 2H, NCH_2), 4.14 (broad s, ∼1H, CH_2), 1.38 (broad s, 1H, OH).

Reaction of the foregoing product (44 mg, 0.20 mmol) with carbon tetrabromide (73 mg, 0.22 mmol) and triphenylphosphine (58 mg, 0.22 mmol) in anhydrous acetonitrile as described above yielded crude (*E*)-1-bromo-4-phthalimido-2-butene-[1,1-$^2H_2$] (not isolated) as a yellow solid. The ^{1}H NMR spectrum indicated ∼56% deuterium incorporation. ^{1}H NMR d 7.87−7.85 (m, 2H, aromatic), 7.74−7.73 (m, 2H, aromatic), 5.95−5.92 (m, 1H, vinylic), 5.87−5.80 (m, 1H, vinylic), 4.31 (d, J = 8.0, 2H, NCH_2), 3.91 (m, ∼1H, CH_2-Br).

The foregoing product reacted with diethyl 2-phthalimidomalonate as described above to give ethyl (*E*)-2-carbethoxy-2,6-diphthalimido-4-hexenoate-[3,3-$^2H_2$] as a white solid. TLC indicated a single compound that comigrated with **8**.

Ethyl (*E*)-2-Carbethoxy-2,6-diphthalimido-4-hexenoate-[3,3,4,5,6,6-$^2H_6$]. The round-bottomed breakseal flask containing gaseous 1,3-butadiene-[$^2H_6$] (1 L at 1 atm, 41 mmol) was cooled to −30 °C, and all the gas was condensed to a liquid. The glass seal was broken, and the outlet was quickly sealed with a rubber septum. After anhydrous chloroform (8 mL) was added, a solution of bromine (6.7 g, 41 mmol) in 5 mL of anhydrous chloroform was added slowly. The mixture was held at −30 °C for 1 h with occasional shaking. After the mixture was warmed to room temperature, the solvent was evaporated under reduced pressure to give (*E*)-1,4-dibromo-2-butene-[$^2H_6$] (not isolated) as a white solid. A small portion of the product (0.5 g) was converted into (*E*)-1-bromo-4-phthalimido-2-butene-[$^2H_6$] (not isolated) by the procedure of Birkofer and Hempel (*17*), and reaction of this with diethyl 2-phthalimidomalonate by the procedure described for **8** gave ethyl (*E*)-2-carbethoxy-2,6-bisphthalimido-4-hexenoate-[3,3,4,5,6,6-$^2H_6$] as a white solid. TLC indicated that the product was a single compound that comigrated with **8**. The ^{1}H NMR spectrum showed only aromatic and ethyl ester signals.

Sample Preparation. Samples were prepared by the procedure of Ballinger et al. (*8*) for observing lysine-based radicals, with modifications. In the earlier work, high concentrations of lysine (0.2 M) were used to ensure that the enzymatic reaction was in the steady-state by the time the samples were frozen and cooled to 77 K. In experiments with *trans*-4,5-dehydrolysine, however, much lower concentrations could be used (10−20 mM) because the compound reacted so slowly that no conversion to 4,5-dehydro-β-lysine could be detected. For this reason, the spectra observed were the same at high as at low concentrations. Samples containing lysine for generating radical ***c*** in Figure 1 were prepared by combining the following in the order specified and to the final concentrations indicated: 0.2 M lysine, 2.3 mM $Na_2S_2O_4$, 1.2 mM AdoMet, and 27 μM reductively incubated 2,3-aminomutase. The sample was mixed, transferred to an EPR tube, and frozen within 35 s by immersion of the tube in liquid N_2. Samples containing *trans*-4,5-dehydrolysine were prepared in the same way, except for the concentrations of the substrate analogue, which were 0.15 M *trans*-4,5-dehydrolysine, 33 mM *trans*-4,5-[2-^{2}H]dehydro-DL-lysine, 24 mM *trans*-4,5-[3,3-$^2H_2$]dehydro-DL-lysine, 33 mM *trans*-4,5-[4,5-$^2H_2$]dehydro-DL-lysine, 25 mM *trans*-4,5-[3,3,4,5,6,6-$^2H_6$]dehydro-DL-lysine, and 50 mM *trans*-4,5-[2,3,3,4,5,6,6-$^2H_7$]dehydro-DL-lysine.

Reversal of 4,5-Dehydrolysyl Radical Formation. All operations were carried out inside the anaerobic chamber. The reaction mixture included 0.24 M Tris-H_2SO_4, 2 mM Na-dithionite, 0.45 mM AdoMet, 77 μM *trans*-4,5-dehydrolysine, and 37 μM 2,3-aminomutase in 0.75 mL. After 20 min at ambient temperature, 250 μL was removed, placed in an EPR tube, and frozen. The remainder was concentrated by ultrafiltration and desalted by gel-filtration through Sephadex G-25 (30 mM Tris-H_2SO_4, pH 8.0, 1 mM DTT, and 10 μM PLP). After the solution was concentrated again, an aliquot (130 μL, 46 μM) was removed, diluted to 250 μL with Tris-H_2SO_4 (0.24 M final concentrated), placed in an EPR tube, and frozen. EPR spectra were recorded on both samples, and the signals were integrated.

EPR Measurements. Low-temperature X-band EPR spectroscopy of $[4Fe\text{-}4S]^+$ bound to 2,3-aminomutase was carried out on a Varian spectrometer equipped with an E102 microwave bridge and an Oxford Instruments ESR-900 continuous flow helium cryostat and an Oxford 3120 temperature controller. Correlative EPR measurements of the 4,5-dehydrolysyl radical at 77 K were carried out on a Varian E-3 spectrometer equipped with a standard liquid nitrogen immersion Dewar. Both spectrometers were interfaced to an IBM microcomputer for data acquisition. Spin concentrations were estimated by double integration of EPR spectra, using either a Varian strong pitch standard (0.1% pitch in KCl) or a 1 mM $CuSO_4$/10 mM EDTA standard. Spin changes of the $[Fe_4S_4]^{1+}$/AdoMet signal were estimated by comparing the area of the trough at g = 1.85 to the trough of an $[4Fe\text{-}4S]^+$/AdoHcy standard that had been quantified in the manner described for the organic radical. All scan conditions for the

$[4Fe\text{-}4S]^+$/AdoHcy species (10 μW power) and the standard were identical.

Time Course of trans-4,5-Dehydrolysine Radical Formation. The study was carried out at ambient temperature (21 ± 2 °C) inside a Coy anaerobic chamber. For each time point, the reaction mixture included 0.28 M Tris-H_2SO_4, 300 μM AdoMet, 460 μM *trans*-4,5-dehydrolysine, 2.8 mM $Na_2S_2O_4$, and 31 μM 2,3-aminomutase in 250 μL contained within EPR tubes. The reactions were initiated by addition of enzyme to the other combined components, and each reaction was quenched at the appropriate time (0.33−5 min) by insertion of the EPR tube into a container of liquid isopentane (−135 °C) placed within a Dewar of liquid nitrogen. The samples were then analyzed by low-temperature EPR for the presence of the $[4Fe\text{-}4S]^+$ and at 77 K for the presence of the 4,5-dehydrolysyl radical as described above.

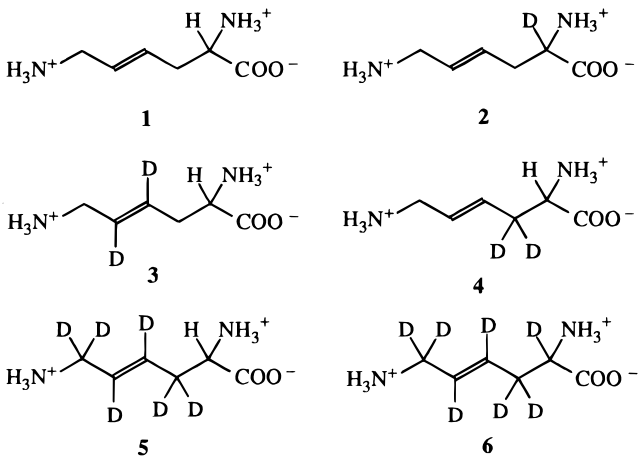
Time Course for Cleavage of AdoMet to 5′-Deoxyadenosine and Methionine. The procedure was carried out through the acid quenching step within the anaerobic chamber. 2,3-Aminomutase was reductively activated by incubation with 132 mM Tris-H_2SO_4 or Epps buffer at pH 8.0, 2.8 mM $Na_2S_2O_4$, 180 μM AdoMet, and 60 μM 2,3-aminomutase in a volume of 250−270 μL. For cleavage to 5′-deoxy[8-^{14}C]-adenosine, [*adenosyl*-8-^{14}C]AdoMet (SA = 3.4×10^6 cpm μmol^{-1}) was used, and for cleavage to [*methyl*-^{14}C]methionine [*methyl*-^{14}C]AdoMet (2.1×10^6 cpm μmol^{-1}) was used. After addition of enzyme to a mixture of the other components of the activation reaction, excess dithionite and AdoMet were removed by gel-filtration on a column of Sephadex G-25 equilibrated in 30 mM Tris-H_2SO_4, 10 μM PLP, and 1 mM DTT. A portion of the activated and desalted enzyme was subjected to spectrophotometric and radiochemical analysis to determine the amount of AdoMet bound to it.

Cleavage of [*adenosyl*-8-^{14}C]AdoMet was initiated by addition of the activated enzyme (100 μL, 10 μM) to a 200-μL solution of 0.36 M Tris-H_2SO_4 at pH 8.0 and 675 μM *trans*-4,5-dehydrolysine. For the $t = 0$ time point, *trans*-4,5-dehydrolysine was excluded. At the designated time (0−5 min), the reaction was terminated with 200 μL of 1 M H_2SO_4. After addition of carrier 5′-deoxyadenosine (40 nmol) and AdoMet (80 nmol) and removal of precipitated protein by centrifugation, one solution was analyzed by HPLC over a Whatman C_{18} column (4.6 × 250 mm) at a flow rate of 1 mL min^{-1}. Elution consisted of a 10-min water wash followed by a linear gradient (0 to 100%) of CH_3CN over 15 min. [*adenosyl*-8-^{14}C]AdoMet emerged in the void volume (2.5 min), and 5′-deoxy[8-^{14}C]adenosine emerged at 21 min.

Cleavage of [*methyl*-^{14}C]AdoMet was initiated by addition of the activated enzyme (80 μM final concentration) to a reaction mixture that included in a final volume of 200 μL 0.24 M Epps at pH 8.0 and 450 μM *trans*-4,5-dehydrolysine. At selected times (0, 10, 30, 60, 180, and 300 s), each reaction was quenched with 48 μL of 2 M perchloric acid. For the $t = 0$ time point, *trans*-4,5-dehydrolysine was excluded. After removal of precipitated protein and neutralization with KOH, the samples were derivatized with PITC and analyzed by HPLC as described (*12*). Fractions (1 mL) were collected and subjected to radiochemical analysis for [*methyl*-^{14}C]AdoMet (25 min) and [*methyl*-^{14}C]methionine (28.5 min).

RESULTS AND DISCUSSION

Synthesis of Deuterium-Labeled trans-4,5-Dehydrolysine. The synthesis of (*E*)-4,5-dehydro-DL-lysine **1** has been reported (*16*, *17*). Deuterated derivatives **2**−**6** were synthesized for the present study.



In the reported synthesis of (*E*)-4,5-dehydro-DL-lysine **1** (*16*, *17*), reaction of (*E*)-1,4-dibromo-2-butene with potassium phthalimide in *N*,*N*-dimethylformamide yielded (*E*)-1-bromo-4-phthalimido-2-butene, which was submitted to reaction with the sodium salt of diethyl acetamidomalonate. The resulting amino acid precursor was hydrolyzed in acid to produce *trans*-4,5-dehydrolysine **1**. A modified procedure using diethyl 2-phthalimidomalonate (*19*) in place of diethyl 2-acetamidomalonate was employed as shown in Scheme 1. Hydrolysis of ethyl (*E*)-2-ethoxycarbonyl-2,6-diphthalimido-4-hexenoate (**8**) afforded *trans*-4,5-dehydrolysine **1**.

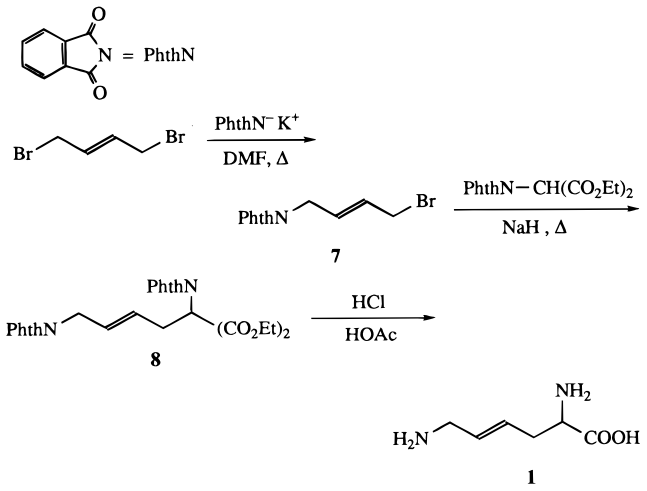
Hydrolysis of **8** or the corresponding labeled analogue in deuterochloric acid and acetic acid-*d* led to the α-deuterated analogues **2** and **6**. Phth^{15}N was used to synthesize 4,5-[6-^{15}N]dehydrolysine.

It is clear from Scheme 1 that (*E*)-1-bromo-4-phthalimido-2-butene **7** is a key intermediate in the synthesis of lysine analogues **1** and **2**. Deuterium-labeled forms of this intermediate were synthesized and employed in the preparation of labeled lysine analogues **3**−**6**. The syntheses of deuterium-labeled analogues of intermediate **7** are described, and all were converted into the deuterated *trans*-4,5-dehydrolysine **3**−**6** by the method in Scheme 1.

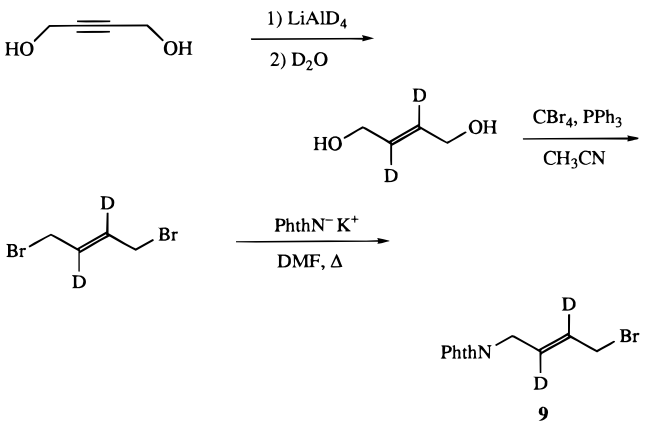
The synthesis of (*E*)-1-bromo-4-phthalimido-2-butene-[2,3-$^2H_2$] began with 2-butyne-1,4-diol as illustrated in Scheme 2. Reaction of 2-butyne-1,4-diol with $LiAlD_4$ followed by addition of D_2O yielded (*E*)-2-butene-1,4-diol-[2,3-$^2H_2$] (*20*). The diol was treated with CBr_4 and triphenyl phosphine in acetonitrile under mild conditions to give (*E*)-1,4-dibromo-2-butene-[2,3-$^2H_2$] (*21*, *22*). Mono-phthalimidation of the dibromide using the same method (*17*) described in Scheme 1 afforded (*E*)-1-bromo-4-phthalimido-2-butene-[2,3-$^2H_2$].

As illustrated in Scheme 3, the synthesis of (*E*)-1-bromo-4-phthalimido-2-butene-[1,1-$^2H_2$] began with methyl 4-bromocrotonate. Diisobutylaluminum hydride (DIBAL) was reported to be an excellent reagent in the selective reduction of an α,β-unsaturated ester to produce the allylic alcohol, and allylic bromide was not reduced under the reaction conditions (*23*). Methyl 4-bromocrotonate was therefore

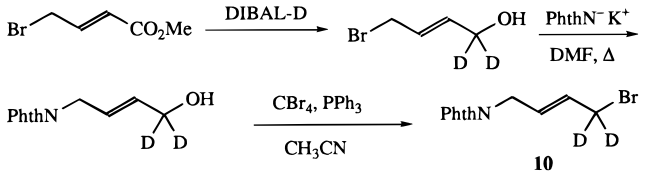
Scheme 1



Scheme 2



Scheme 3

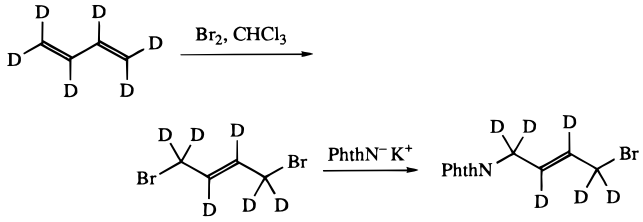


reduced with diisobutylaluminum deuteride (50% D) to yield (*E*)-4-bromo-2-butene-1-ol-[1,1-$^2H_2$] (*23*). Treatment of the bromo-alcohol with potassium phthalimide by the method of Sheehan and Bolhofer (*18*) gave (*E*)-4-phthalimido-2-butene-1-ol-[1,1-$^2H_2$], which was converted into (*E*)-1-bromo-4-phthalimido-2-butene-[1,1-$^2H_2$] by the procedure in Scheme 2.

The perdeutero-intermediate (*E*)-1-bromo-4-phthalimido-2-butene-[$^2H_6$] was prepared from 1,3-butadiene-[$^2H_6$] as shown in Scheme 4. The 1,4-addition of bromine by the procedure of Shantz (*24*) produced (*E*)-1,4-dibromo-2-butene-[$^2H_6$]. Mono-phthalimidation of the dibromide as described in Scheme 1 gave (*E*)-1-bromo-4-phthalimido-2-butene-[$^2H_6$].

Inhibition by trans-4,5-Dehydrolysine. Conventional tests for inhibition of lysine-2,3-aminomutase invariably showed potent inhibition, suggesting an inhibition constant in the range of $\leq$100 μM. Inhibition was blocked by high concentrations of lysine ($\geq K_m$), indicating competition. However,

Scheme 4



good inhibition data in conventional experiments could not be obtained because the system behaved as if the enzyme were being irreversibly inhibited by *trans*-4,5-dehydrolysine. EPR experiments described in a later section clarified the interaction of *trans*-4,5-dehydrolysine with this enzyme by showing that its reaction required 5−10 min to attain an equilibrium state at saturating inhibitor concentrations. The EPR experiments also indicated a value of $K_d < 50$ μM.

trans-4,5-Dehydrolysine is an analogue of the substrate L-lysine, and the question of whether it reacts as an alternative substrate was investigated in assays employing HPLC or NMR for product detection. While an authentic sample of 4,5-dehydro-β-lysine was not available for definitive comparison, it was expected that its appearance in reaction mixtures of *trans*-4,5-dehydrolysine with lysine-2,3-aminomutase would be detected in the NMR spectra and on HPLC elution profiles. In no case could a product be detected under conditions in which the isomerization of lysine to β-lysine went to equilibrium. It was concluded that *trans*-4,5-dehydrolysine either was not a substrate or its turnover number was too low to be observed in conventional experiments.

EPR experiments, described in a later section, verified that the reaction of *trans*-4,5-dehydrolysine with 2,3-aminomutase is slow and reaches equilibrium in 5−10 min. When compared with the enzymatic turnover number of about 30 s^{-1} at 37 °C, the results indicated that a turnover number for *trans*-4,5-dehydrolysine would be <0.015 s^{-1}. Thus, *trans*-4,5-dehydrolysine behaves, for practical purposes, as an irreversible inhibitor. That its reaction takes place at the active site is strongly indicated by its competition with lysine and the requirement for AdoMet for radical-formation reaction, as described in the following section.

Radical Formation in the Reaction of trans-4,5-Dehydrolysine with Lysine 2,3-Aminomutase. The EPR spectrum of a new organic radical is observed when *trans*-4,5-dehydrolysine is substituted for lysine in experiments with 2,3-aminomutase. Shown in Figure 2 are the EPR spectra of the product-related radical generated from lysine (Figure 2A) and of the new radical generated from *trans*-4,5-dehydrolysine (Figure 2B). The new radical appears slowly, as detailed in a later section, and attains a high concentration, corresponding to virtually all of the functionally active sites in 2,3-aminomutase.

The new radical appears to be the allylic species resulting from abstraction of a hydrogen atom from C3 of the external aldimine of *trans*-4,5-dehydrolysine with PLP. Evidence supporting this interpretation consists of the EPR spectral changes brought about by the use of variously deuterated *trans*-4,5-dehydrolysine for generating the radical. Representative EPR spectra are shown in Figure 3. Replacement of hydrogen with deuterium at any carbon of *trans*-4,5-

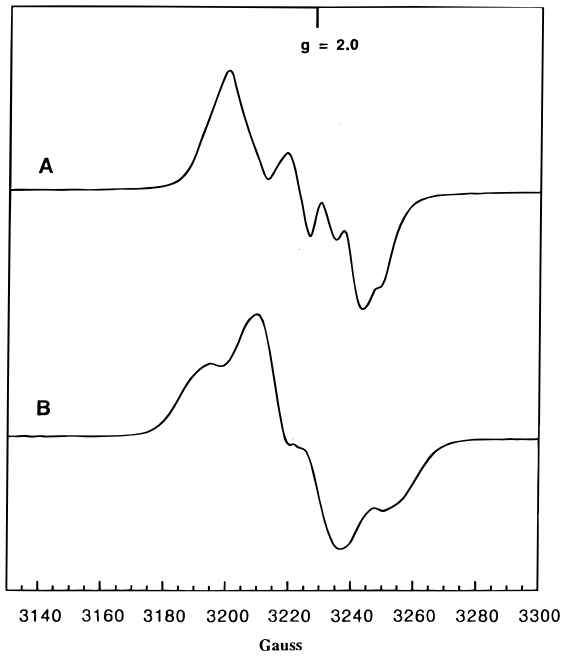


FIGURE 2: EPR spectra of lysyl and 4,5-dehydrolysyl radicals at the active site. Active lysine 2,3-aminomutase was mixed with lysine or *trans*-4,5-dehydrolysine in the anaerobic chamber and transferred to an EPR tube, and the tube was immersed in liquid N_2 within 35 s. The solutions contained 0.15 M lysine (part A) or *trans*-4,5-dehydrolysine (part B), 1.2 mM AdoMet 2.3 mM $Na_2S_2O_4$, 50 mM Tris-sulfate buffer at pH 8.0, and lysine 2,3-aminomutase. Spectra were recorded at 9.05 GHz with a modulation amplitude of 1.6 G at 4 mW power, with gains set at 1.5×10^4. The spectra were obtained at 27 μM (part A) or 45 μM (part B) lysine 2,3-aminomutase and normalized to 25 μM enzyme. Spectra shown are computed averages of four 4-min scans with a time constant of 0.3 s. The spectrum in part A is typical of the previously characterized lysyl radical ***c*** in Figure 1 (*7*, *8*, *10*). The spectrum in part B is shown in Figure 3 to be of an allylic analogue of lysyl radical ***a*** in Figure 1, produced by abstraction of a hydrogen atom from C3 of the external aldimine of *trans*-4,5-dehydrolysine with PLP at the active site.

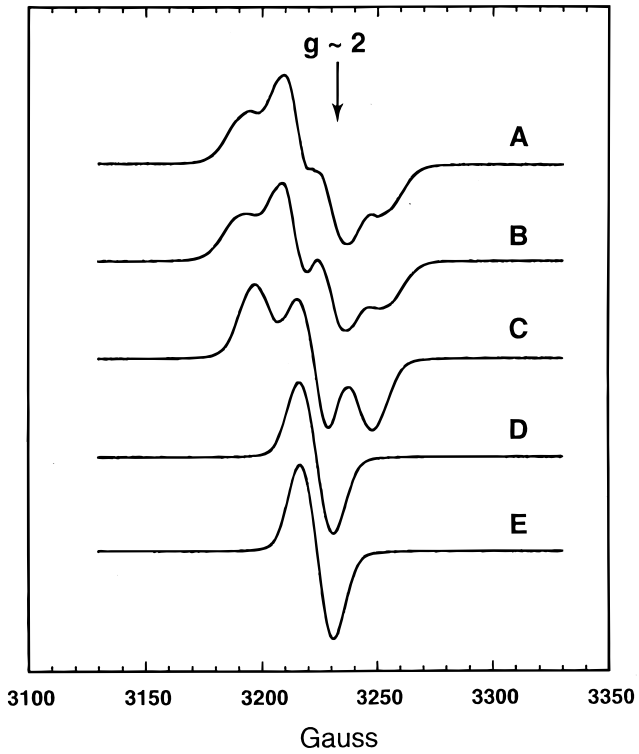


FIGURE 3: EPR spectra of the 4,5-dehydrolysyl radicals obtained with deuterium-labeled *trans*-4,5-dehydrolysine. Reaction mixtures and procedure were as described in Figure 2, with 45 μM lysine 2,3-aminomutase and the following forms of *trans*-4,5-dehydrolysine: (A) unlabeled *trans*-4,5-dehydrolysine; (B) *trans*-4,5-[2-^{2}H]dehydrolysine; (C) *trans*-4,5-[4,5-$^2H_2$]dehydrolysine; (D) *trans*-4,5-[3,3,4,5,6,6-$^2H_6$]dehydrolysine; (E) *trans*-4,5-[2,3,3,4,5,6,6-$^2H_7$]dehydrolysine. A spectrum (not shown) was also obtained with *trans*-4,5-[3,3-$^2H_2$]dehydrolysine. All spectra were accumulated from four scans (4 min/scan), and the microwave frequency was 9.045 GHz.

dehydrolysine alters the EPR spectrum of the radical, proving that the unpaired electron resides on the carbon skeleton of this inhibitor. Moreover, the fact that deuterium substitution at C2 or C6 significantly perturbs the spectrum implies that spin density resides at both C3 and C5, the allylic carbon atoms. More significant changes in the spectra are obtained by replacing the hydrogens bonded to C4 and C5 with deuterium (Figure 3C), and a similar change is seen with *trans*-4,5-[3,3-$^2H_2$]dehydrolysine (spectrum not shown). Deuterium substitution at all positions collapses the residual hyperfine splitting (Figure 3E). Thus, a qualitative inspection of the spectra in Figure 3 leads immediately to the preliminary assignment of the 4,5-allylic analogue of the substrate-related radical ***a*** in Figure 1.

The spectra obtained with *trans*-4,5-[2-^{2}H]dehydrolysine (Figure 3B) and *trans*-4,5-[4,5-$^2H_2$]dehydrolysine (Figure 3C) were chosen for more detailed analysis. Computer simulations of these spectra were undertaken to determine the conformation of the radical. To facilitate simulation, the spectra were resolution-enhanced to obtain estimates of the hyperfine splitting constants for the interactions of the unpaired electron with nearby nuclei, and these values were refined by the simulations. The parameters leading to the best simulations attained are listed in Table 1. They identify

Table 1: Hyperfine Splitting Parameters Obtained from Spectral Simulations of the Resolution-Enhanced EPR Spectra[a]

	principle values of hyperfine tensor (**G**)			
nucleus	A_{xx}	A_{yy}	A_{zz}	a_o (G)
^{1}H3	27	8.4	18	17.8 ± 0.9
^{1}H4	5.7	4.1	2.4	4.1 ± 0.6
^{1}H5	16.7	5.2	11.5	11.1 ± 0.6
^{1}H6	18.4	18.4	18.4	18.4 ± 1.0
^{14}N2	5.8	5.8	5.8	5.8 ± 0.5

[a] The principal values of the **g** tensor are: $g_{xx} = 2.0036$, $g_{yy} = 2.0036$, and $g_{zz} = 2.0021$.

the species as an allylic radical derived from *trans*-4,5-dehydrolysine, with significant spin density on C3 and C5. The resolution-enhanced spectra and simulations are shown in Figure 4.

Overlap of *p*-orbitals of the carbon atoms in an allylic system distributes spin density over these orbitals, with a node of spin density on the middle carbon, C4 in the present case. This spin distribution has been demonstrated experimentally in the structure of the glutaconic acid radical in a single crystal study by Heller and Cole (*25*) and in a study of the allyl radical in the gas phase (*26*). The unpaired spin density in the *p*-orbital of each carbon atom interacts with the nuclear spin of the protons attached to these carbon atoms and, to variable degrees depending on dihedral angles, with protons bonded to adjacent carbon atoms. Therefore, it is

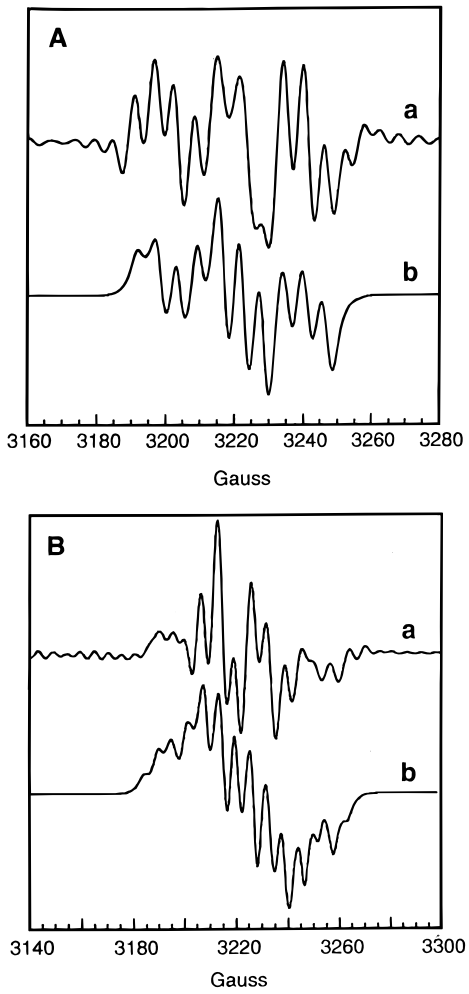


FIGURE 4: Comparison of resolution-enhanced experimental spectra with simulated spectra. Spectra analyzed from Figure 3 were of (A) *trans*-4,5-[4,5-$^2H_2$]dehydrolysine and (B) *trans*-4,5-[2-^{2}H]-dehydrolysine. Resolution-enhanced spectra were obtained (panel A, part a) $\sigma = 8$ G and $L = 0.225$ G^{-1} and (panel B, part b) $\sigma = 10$ G and $L = 0.225$ G^{-1} using a squared Bartlett window function, $(1 - |x|/L)^2$. Simulations were obtained using $g_{xx} = 2.0036$, $g_{yy} = 2.0036$, $g_{zz} = 2.0021$. The simulations of the *trans*-4,5-[4,5-$^2H_2$]-dehydrolysine (panel A, part b) included the ^{14}N2, ^{1}H3, ^{1}H4, ^{1}H5, ^{1}H6, and ^{14}N6. The hyperfine splitting constants were as listed in Table 1. Line widths were (panel A, part a) 2.25 G and (panel B, part b) 2 G; 625 crystal orientations were sampled in the simulations.

possible to determine the conformation of a radical by examining the strengths of the hyperfine interactions with nearby nuclei, as expressed in the values of the splitting constants.

The hyperfine splitting constants between the unpaired electron and the protons bonded to C3, C4, and C5, given in Table 1, show that the spin density is unevenly distributed. In this allylic radical generated at an enzymatic site, about 60% of the unpaired spin density resides on C3.

Conformation of the 4,5-Dehydrolysyl Radical. Allylic radicals can adopt a number of conformations in solution. The most stable for the side chain of *trans*-4,5-dehydrolysine is one in which C3, C4, and C5 lie in one plane with their p-orbitals perpendicular to that plane. In an enzymatic active site, however, the conformation of a molecule is generally dictated by the conformation of the binding site and the mechanisms of catalytic steps. Therefore, the orientations

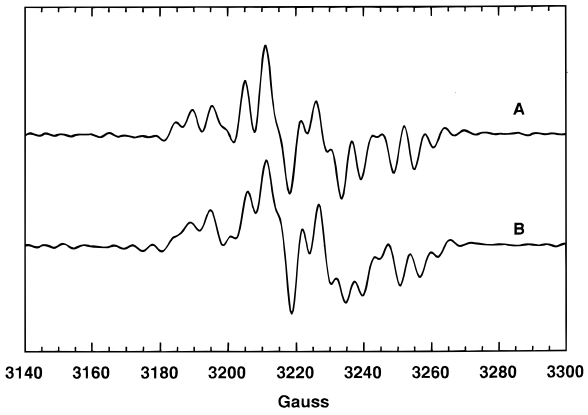


FIGURE 5: Comparison of EPR spectra obtained with *trans*-4,5-[6-^{15}N]dehydrolysine (A) and *trans*-4,5-dehydrolysine (B). Resolution-enhanced spectra were obtained (A,a) $\sigma = 9$ G and $L = 0.225$ G^{-1} and (B,b) $\sigma = 8$ G and $L = 0.225$ G^{-1} using a squared Bartlett window function, $(1 - |x|/L)^2$. Other conditions are given in the legend for Figure 2.

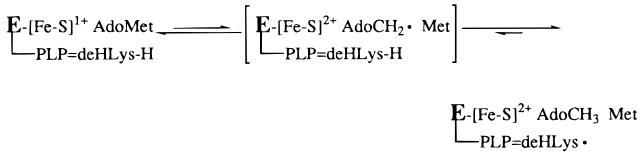
of carbon atoms will not necessarily correspond to the most stable conformation for a radical, especially when a radical is generated from a substrate analogue, as in the present case.

While the distribution of unpaired spin may be different in an enzyme-bound radical than in solution or in single crystals, the total spin must be the same. One measure of the total spin is the sum of the isotropic splitting constants for protons attached to the first and third carbon atoms of an allylic system. This sum for the allylic radical in the gas phase is 29.1 G (*26*). In the 4,5-dehydrolysine radical bound to 2,3-aminomutase, the sum of isotropic components is 28.9 G, in agreement with the allylic radical (*26*). The sum of isotropic components for the glutaconic acid radical is 25.5 G (*25*), which is also in good agreement after correction for the further delocalization of spin density into the carboxylic acid group of this radical.

Interactions of the unpaired spin at C3 and C5 with substituents on adjacent carbon atoms provide information on the conformation of the radical (*8*). In the present case of the allylic analogue of the substrate-related radical ***a*** (Figure 1), hyperfine splitting with ^{14}N could involve either the α-^{14}N or the ϵ-^{14}N engaged in an external aldimine linkage with PLP. To determine whether there was hyperfine splitting between the unpaired electron and the ϵ-^{14}N of *trans*-4,5-dehydrolysine, *trans*-4,5-[6-^{15}N]dehydrolysine was synthesized and used to generate the radical. The resulting spectrum was subjected to resolution-enhancement and compared with that of a matched sample from unlabeled *trans*-4,5-dehydrolysine. As shown in Figure 5, the spectra are indistinguishable, which rules out hyperfine splitting from the ϵ-^{14}N. Therefore, the observed ~6 G triplet splitting is assigned to ^{14}N of the α-amino group.

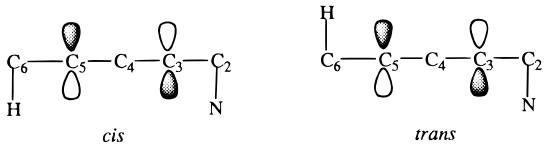
Hyperfine splitting from protons depends on the dihedral angle between the p-orbial and the proton through the relationship $A = \rho(B + C\cos^2\theta)$, where ρ is the spin density, B and C are empirically determined constants (0.92 and 42 G, respectively), and θ is the dihedral angle (*27*). The splitting between the C2(H) and the unpaired spin density at C3 was small and not measured directly. The nearly eclipsed orientation of the α-nitrogen with respect to the p-orbital on C3 forces the C2(H) to adopt a dihedral angle leading to a minimal interaction with the unpaired spin

Scheme 5



density at C3 (70−90°). In contrast, one of the protons C6-(H) strongly interacts with the unpaired spin density at C5. On the basis of the splitting constant (18.5 G) and the spin density on C5 (∼ 40%), it can be estimated that this proton is nearly eclipsed with the p-orbital of C5.

The orientations of the substituents on C6 relative to those on C2 remain unresolved. The two possibilities are illustrated below, with either the *cis*- or *trans*- orientations being allowed equally well by the data.



Correlation of Radical Formation with AdoMet Cleavage. Preliminary rate experiments revealed that the inhibition of 2,3-aminomutase by *trans*-4,5-dehydrolysine was a slow process that required several minutes to reach completion. This time course presented a means by which the working hypothesis in Scheme 5 for the mechanism of radical formation could be critically tested.

According to the hypothesis, the reversible cleavage of AdoMet to methionine and the 5′-deoxyadenosyl radical is accompanied by the reversible oxidation of the EPR-active $[4Fe\text{-}4S]^{+}$ to the EPR-silent $[4Fe\text{-}4S]^{2+}$ (*6*). The process is not normally thermodynamically favored because of the high energy of the 5′-deoxyadenosyl radical. However, in the presence of a substrate or substrate analogue, hydrogen abstraction by 5′-deoxyadenosyl radical proceeds to the formation of observable substrate radicals (*8*, *9*). The highly stabilized 4,5-dehydrolysyl radical draws the equilibrium of Scheme 5 to essential completion, and the process is slow enough to be monitored with time.

If the mechanism of Scheme 5 is valid, the rate at which the 4,5-dehydrolysyl radical appears (dehydroLys• in Scheme 5) must be the same as the rate at which $[4Fe\text{-}4S]^{+}$ disappears by transformation into the EPR-silent $[4Fe\text{-}4S]^{2+}$. These rates have been estimated from the data in Figure 6, where the intensity of the low-temperature EPR signal for $[4Fe\text{-}4S]^{+}$ as a function of time is plotted together with the increase in the intensity of the EPR signal for the 4,5-dehydrolysyl radical in the same reaction mixture. The signal for $[4Fe\text{-}4S]^{+}$ decreases on the same time scale as the increase in the signal for the 4,5-dehydrolysyl radical. The rate constants for the two processes are 2.6 ± 0.4 and 2.9 ± 0.6 min^{-1}, respectively, under the experimental conditions. These rate constants are identical within error, which strongly supports the mechanism.

A further characteristic of the mechanism in Scheme 5 is that AdoMet is cleaved to 5′-deoxyadenosine and methionine concomitant with the formation of the 4,5-dehydrolysyl radical. Such a cleavage of AdoMet has been described (*28*). However, Scheme 5 requires that the rate of this cleavage must be equal to or greater than the rate of radical formation. The rates of cleavage to 5′-deoxyadenosine and methionine are shown in Figure 7, panels A and B, respectively, under conditions similar to those in Figure 6 for radical formation. The rate constants are approximately 5 min^{-1}, similar to or somewhat faster than the rate constant for radical formation, further supporting the radical initiation mechanism.

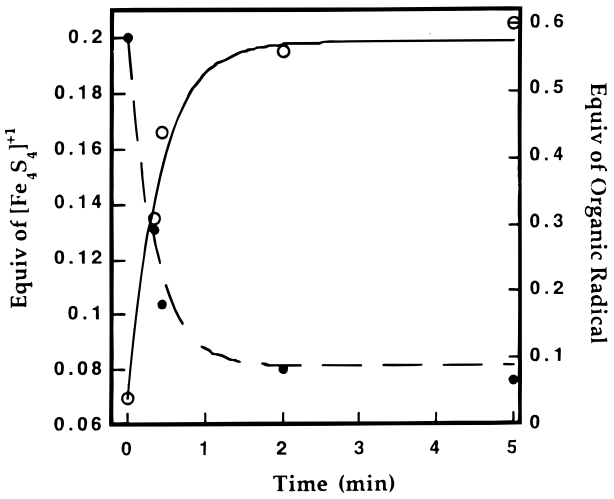


FIGURE 6: Time course for the formation of the 4,5-dehydrolysyl radical and decrease of the $[Fe_4S_4]^{1+}$ EPR signal. Closed circles represent the equivalents of $[Fe_4S_4]^{1+}$ species per holoenzyme. Open circles represent the equivalents of *trans*-4,5-dehydrolysine radical per holoenzyme. Fits to a single-exponential function give rate constants of 2.9 ± 0.6 min^{-1} for formation of the organic radical and 2.6 ± 0.4 min^{-1} for loss of the $[Fe_4S_4]^{1+}$ species. Detailed procedure is described in Experimental Procedures.

Stability of the 4,5-Dehydrolysyl Radical at the Active Site. The 4,5-dehydrolysyl radical is slowly generated (k = 3 min^{-1}) and is stable at the active site of lysine 2,3-aminomutase. Its stability may be due in large part to allylic stabilization; however, its stability should also enhance the rate of its formation relative to the rate at which the corresponding lysyl radical is generated from L-lysine, which is ≥24 s^{-1}, the value of k_{cat} (*11*). Therefore, slow formation of the 4,5-dehydrolysyl radical must be explained by differences in the interactions of the active site with 4,5-dehydrolysine and L-lysine. These differences could affect the rates of any or all steps leading up to hydrogen abstraction from C3, which include binding and imine formation with PLP. The rigid *trans*-butenyl segment of *trans*-4,5-dehydrolysine may also impose a steric constraint on the process of hydrogen abstraction from C3 that could slow the rate of 4,5-dehydrolysyl radical formation. In any case, the 4,5-dehydrolysyl radical is a structural analogue of the intermediate substrate radical ***a*** in Figure 1. Its slow formation provides leverage for a critical test of the mechanism of radical initiation, which is supported by the present results.

Although the 4,5-dehydrolysyl radical is structurally analogous to radical ***a*** (Figure 1) in the reaction of L-lysine, it does not react further to produce 4,5-dehydro-β-lysine. To determine whether it reacts in reverse to re-form *trans*-4,5-dehydrolysine, the radical was generated by using [*adenosyl*-2,8,5′-^{3}H]AdoMet in place of AdoMet. After 30 min, the radical-containing complex was denatured, and *trans*-4,5-dehydrolysine was reisolated and found to contain no detectable tritium. If the 4,5-dehydrolysyl radical had been transformed back to *trans*-4,5-dehydrolysine in this experi-

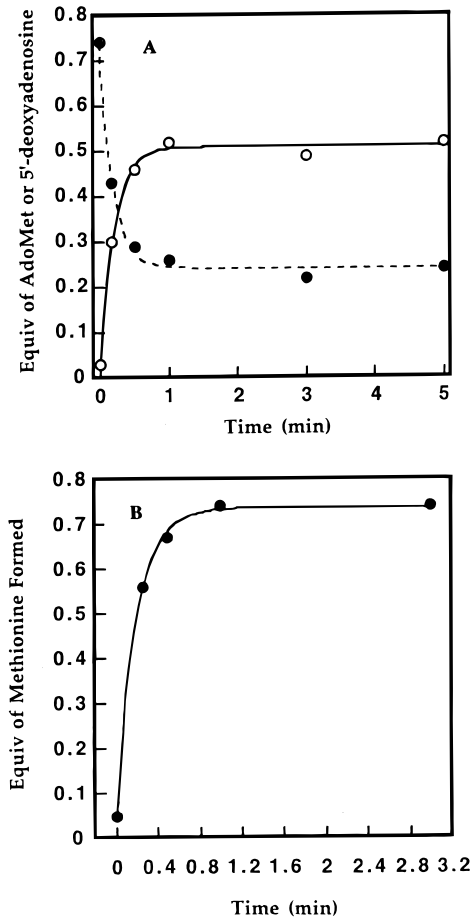


FIGURE 7: Time course for the cleavage of AdoMet to 5′-deoxyadenosine and methionine. Panel A: Closed circles represent the equivalents of AdoMet per holoenzyme. Open circles represent the equivalent of 5′-deoxyadenosine per enzyme. The $[Fe_4S_4]^{1+}$ species was generated with Na-dithionite and [*adenosyl*-8-^{14}C]-AdoMet. Upon gel-filtration, 0.75 equiv of [*adenosyl*-8-^{14}C]AdoMet were bound to the enzyme. Addition of *trans*-4,5-dehydrolysine resulted in a time-dependent formation of 5′-deoxy[8-^{14}C]adenosine with concomitant loss of [*adenosyl*-8-^{14}C]AdoMet. Data were fitted to a single-exponential function and gave rate constants of 4.9 ± 0.5 and 5.4 ± 0.62 min^{-1} for loss of [*adenosyl*-8-^{14}C]AdoMet and formation of 5′-deoxy[8-^{14}C]adenosine. Panel B: The $[Fe_4S_4]^{1+}$ species was generated with Na-dithionite and [*methyl*-^{14}C]AdoMet. Upon gel-filtration, 1.1 equiv of [*methyl*-^{14}C]AdoMet were bound to the enzyme. Addition of *trans*-4,5-dehydrolysine resulted in a time-dependent formation of [*methyl*-^{14}C]methionine. Data were fitted to a single-exponential function and gave a rate constant of 5.2 ± 0.4 min^{-1}. Detailed procedures are described in the Experimental Procedures.

ment, it should have acquired tritium from the 5′-carbon of [*adenosyl*-2,8,5′-^{3}H]AdoMet. However, no tritium could be detected under conditions in which all of the tritium at C5′ was transferred to lysine and β-lysine within 1 min in a control experiment with L-lysine as the substrate.[2] Therefore, neither the forward nor the reverse reaction of the 4,5-dehydrolysyl radical could be detected. Evidently, the allylic radical is too stable to react significantly within 30 min. It should be regarded as a mechanism-based inactivator of lysine 2,3-aminomutase, in which the stable, inactivated enzyme complex is blocked from further reaction by the stability of an allylic radical analogous in structure to the substrate-related radical intermediate in the reaction of L-lysine.

[2] Unpublished results of S. Booker and P. A. Frey.

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