Electron Transfer in the Substrate-Dependent Suicide Inactivation of Lysine 5,6-Aminomutase†

Kuo-Hsiang Tang,‡ Christopher H. Chang,‡ and Perry A. Frey*

Department of Biochemistry, University of Wisconsin−Madison, 1710 University Avenue, Madison, Wisconsin 53705

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ABSTRACT: The lysine 5,6-aminomutase (5,6-LAM) purified from *Clostridium sticklandii* was found to undergo rapid inactivation in the absence of the activating enzyme E_2 and ATP. In the presence of substrate, inactivation was also seen for the recombinant 5,6-LAM. This adenosylcobalamin-dependent enzyme is postulated to generate cob(II)alamin and the 5′-deoxyadenosyl radical through enzyme-induced homolytic scission of the Co−C bond. However, the products cob(III)alamin and 5′-deoxyadenosine were observed upon inactivation of 5,6-LAM. Cob(III)alamin production, as monitored by the increase in A_{358}, proceeds at the same rate as the loss of enzyme activity, suggesting that the activity loss is related to the adventitious generation of cob(III)alamin during enzymatic turnover. The cleavage of adenosylcobalamin to cob(III)-alamin is accompanied by the formation of 5′-deoxyadenosine at the same rate, and the generation of cob(III)alamin proceeds at the same rate both aerobically and anaerobically. Suicide inactivation requires the presence of substrate, adenosylcobalamin, and PLP. We have ruled out the involvement of either the putative 5′-deoxyadenosyl radical or dioxygen in suicide inactivation. We have shown that one or more reaction intermediates derived from the substrate or/and the product, presumably a radical, participate in suicide inactivation of 5,6-LAM through electron transfer from cob(II)alamin. Moreover, L-lysine is found to be a slowly reacting substrate, and it induces inactivation at a rate similar to that of D-lysine. The alternative substrate β-lysine induces inactivation at least 25 times faster than DL-lysine. The inactivation mechanism is compatible with the radical isomerization mechanism proposed to explain the action of 5,6-LAM.

Clostridium sticklandii ferment DL-lysine to acetic acid, butyric acid, and ammonia (*1*). Two pathways of lysine catabolism in this bacterium differ for L- and D-lysine (*2*). Lysine 5,6-aminomutase (5,6-LAM)[1] participates in both pathways to move the ϵ-amino group of D-lysine or of β-L-lysine from C6 to C5 of the side chain (*3*, *4*), facilitating eventual oxidative cleavage.

The dual activities of 5,6-LAM require two protein components, the core enzyme E_1 and an activating protein E_2 (*1*). E_1 appears to be a heterotetramer composed of α- and β-subunits and formulated as $\alpha 2\beta 2$, the molecular mass of which is approximately 170 kDa. The molecular masses of the β- and α-subunits are 30 and 55 kDa, respectively (*5*). The amino acid sequence predicted from the nucleotide sequence of the gene includes a conserved adenosylcobalamin binding motif with a histidine residue displacing the lower axial dimethylbenzimidazole ligand, and a 3-cysteine cluster in the small subunit, as well as a P-loop sequence in the large subunit (*6*). Previous EPR results for 5,6-LAM with [^{15}N](dimethylbenzimidazole)adenosylcobalamin demonstrated base-off binding, consistent with other B_{12}-dependent enzymes that break unactivated C−H bonds (*6*). These enzymes replace the dimethylbenzimidazole moiety with the imidazole ring of a histidine residue as the lower axial ligand to cobalt(III). Enzymes, such as ribonucleotide reductase (*9*), diol dehydrase (*10*, *11*), and ethanolamine ammonia lyase (*12*), bind adenosylcobalamin with the dimethylbenzimidazole moiety bonded to cobalt(III) as the lower axial ligand. This type of adenosylcobalamin binding is known as "base-on". The latter enzymes, in contrast to the base-off type, initiate catalysis by abstracting a hydrogen atom from a heteroatom or a carbon attached to a heteroatom.

The forward reaction catalyzed by 5,6-LAM acting on D-lysine is a 1,2-migration of the ϵ-amino group to the δ-carbon, with concomitant reverse migration of a hydrogen atom, to produce 2,5-DAH (*1*, *2*). The reaction is reversible and likely to display an equilibrium constant near 1. 5,6-LAM activity requires both adenosylcobalamin and PLP (*13*, *14*). The overall reaction requires cleavage of unreactive

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* To whom correspondence should be addressed: University of Wisconsin−Madison, 1710 University Ave., Madison, WI 53705. Telephone: (608) 262-0055. Fax: (608) 265-2904. E-mail: frey@biochem.wisc.edu.

‡ These authors contributed equally to the research in this paper.

[1] Abbreviations: 5,6-LAM, lysine 5,6-aminomutase; ATP, adenosine 5′-triphosphate; β-ME, β-mercaptoethanol; dIH_2O, doubly distilled water; 2,5-DAH, 2,5-diaminohexanoic acid; DTT, dithiothreitol; PITC, phenyl isothiocyanate; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DL-lysine-d_8, DL-[3,3,4,4,5,5,6,6-$^2H_8$]lysine; EPR, electron paramagnetic resonance spectroscopy; ESI-MS, electrospray ionization spectrometry; HPLC, high-pressure liquid chromatography; LC−MS, reversed phase liquid chromatography−mass spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance; PLP, pyridoxal 5′-phosphate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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C−H and C−N bonds, both of which are strong and present high energy barriers to cleavage. The K_m values for adenosylcobalamin and PLP for the recombinant enzyme are 6.6 and 1.0 μM, respectively (*6*).

Adenosylcobalamin is the coenzyme for a family of enzymatic reactions (*7*−*12*), which share in common the 1,2-interchange of a hydrogen atom with an adjacent group. The generally accepted mechanisms of these reactions involve common steps, which include (a) enzyme-induced homolytic scission of the Co−C bond to cob(II)alamin and the 5′-deoxyadenosyl radical, (b) hydrogen abstraction from the substrate by the 5′-deoxyadenosyl radical, (c) isomerization of the resulting substrate radical to the product radical, (d) transfer of a hydrogen atom from 5′-deoxyadenosine to the product radical to produce the product and regenerate the 5′-deoxyadenosyl radical, and (e) recombination of cob(II)alamin with the 5′-deoxyadenosyl radical to complete one turnover. The bond dissociation energy of the Co−C bond in free adenosylcobalamin has been reported to be approximately 30 kcal/mol (*16*, *21*). Cleavage of the Co−C bond is thought to be facilitated by steric effects associated with enzyme-induced conformational distortion of the corrin ring.

The mechanism of the reaction of 5,6-LAM is expected to be analogous to that of the *S*-adenosyl-L-methionine and PLP-dependent L-lysine 2,3-aminomutase from *Clostridium subterminale* (*15*) with an important exception. Transient generation of the 5′-deoxyadenosyl radical through the concerted reaction between the [4Fe-4S] cluster and *S*-adenosyl-L-methionine in the reaction of L-lysine 2,3-aminomutase is postulated to be replaced by homolytic scission of the Co−C bond of adenosylcobalamin in the reaction of 5,6-LAM. A radical mechanism for 1,2-amino group migration has been documented by spectroscopic observations in the reaction of L-lysine-2,3-aminomutase (*17*). This is the inspiration for the hypothetical mechanism in Figure 1 for the reaction of 5,6-LAM. The postulated role of adenosylcobalamin is, as in other coenzyme B_{12}-dependent reactions, to generate the 5′-deoxyadenosyl radical, which abstracts a hydrogen atom from C5 of D-lysine. The substrate is presumed to be bound at the active site in the form of an external aldimine with PLP. The substrate radical intermediate **2** undergoes internal addition to the imine N to form the aziridylcarbinyl-PLP adduct **3**. By cleavage of the C6−N bond in a fashion similar to that of cyclopropyl carbinyl radical clocks (*18*), the chemistry moves forward to the product radical **4**. Abstraction of a hydrogen atom from the methyl group of 5′-deoxyadenosine by radical **4** completes the rearrangement of the amino group and regenerates the 5′-deoxyadenosyl radical, which may recombine with cob(II)alamin for another round of catalysis.

The 5,6-LAM purified from *C. sticklandii* loses activity in the absence of the activating enzyme E_2 and ATP (*5*). The recombinant 5,6-LAM produced by expression of the *kamD* and *kamE* genes in *Escherichia coli* is subject to substrate-induced suicide inactivation (*6*), a process that also requires adenosylcobalamin and PLP. In this report, we trace the course of hydrogen transfer in suicide inactivation. We present a mechanism for inactivation that is compatible with the proposed catalytic mechanism in Figure 1.

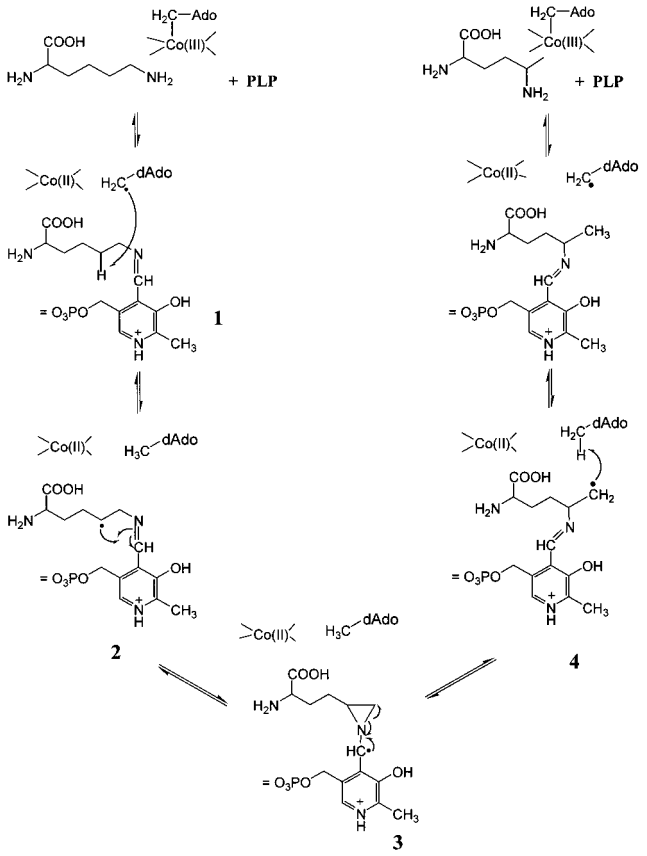


FIGURE 1: Proposed mechanism of action of lysine 5,6-aminomutase. The mechanism we proposed is derived in analogy to that for L-lysine 2,3-aminomutase. The similarity and analogy in cofactor requirements of these two enzymes, PLP and a potential radical-generating species, support this hypothesis.

MATERIALS AND METHODS

Materials. D-[1-^{14}C]Lysine, [U-^{14}C]PLP, and ^3H_2O were from ARC. L-[U-^{14}C]Lysine was obtained from NEN Life Science Products. DL-[3,3,4,4,5,5,6,6-$^2H_8$]Lysine (98%) and L-[3,3,4,4,5,5,6,6-$^2H_8$]lysine (98%) were from Isotec. 5′-Deoxyadenosine, D-lysine, L-lysine, adenosylcobalamin, PLP, β-ME, protocatechuate, and protocatechuate dioxygenase were from Sigma. β-Lysine was synthesized enzymatically from L-lysine with L-lysine 2,3-aminomutase (*23*). ^2H_2O, trifluoroacetic acid, and trichloroacetic acid were from Aldrich. C_{18} and silica Sep-Pak cartridges were purchased from Millipore. Nonfluorescent thin-layer chromatography plates on silica gel G were from Whatman. The recombinant E_1 component of 5,6-LAM from *C. sticklandii* was produced by expression in *E. coli* from Clostridial genes *kamD* and *kamE* and purified as previously described (*6*). J. Escalante generously supplied adenosylcobalamin synthetase (CobA). The strain of ribonucleotide reductase (RTPR), HB101/pSQUIRE, for expression of RTPR was the generous gift of J. Stubbe (Department of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, MA). The enzyme was expressed and purified as previously reported (*19*).

Analytical Methods. Standard and kinetic UV−visible spectra were measured on a Hewlett-Packard model 8452A diode array spectrophotometer with the cuvette holder temperature maintained by a circulating water bath. The amount of radioactivity was measured by liquid scintillation

counting in a Beckman LS 6500 scintillation system. The inactivation rates by β-lysine and D-lysine were collected using an OLIS Inc. RSM-1000 stopped-flow spectrophotometer. Mass spectra were collected either on a Perkin-Elmer Sciex API 365 triple-quadrupole ESI mass spectrometer (ESI-MS) at the University of Wisconsin Biotechnology Center (Madison, WI) or on an HP 1100 series LC MSD apparatus (LC−MS) using atmospheric pressure electrospray ionization in the positive ion mode. 5,6-LAM was assayed as described previously (*6*). Acid quenching assays were analyzed by thin-layer chromatography with the mobile phase being a 40:40:15 $CH_3OH/CHCl_3$/12% NH_4OH mixture. The amount of ^{14}C radioactivity of the substrate and product was counted in a Beckman LS 6500 scintillation system.

Equilibrium Constant for Conversion of D-Lysine into 2,5-DAH. 5,6-LAM (100 μM) was incubated at 37 °C for 3−4 min with 100 mM NH_4EPPS buffer (pH 8.5), 5 mM β-ME, 100 μM adenosylcobalamin, and PLP in 225 μL. The reactions were initiated by addition of 100 mM D-[1-^{14}C]-lysine (25 μL of a 1 M stock). Timed aliquots (5 μL) were withdrawn and quenched with 2 μL of 2 N $HClO_4$. After the protein precipitate had been removed by low-speed centrifugation, the supernatant fluid was analyzed by thin-layer chromatography on silica gel G (Whatman) and radiochemical analysis for product formation and residual substrate. The product ($R_f = 0.30$) migrated ahead of the substrate ($R_f = 0.15$) with a 40:40:15 $CH_3OH/CHCl_3$/12% NH_4OH mixture as the mobile phase. The amount of radioactivity of the product and substrate spots was measured by liquid scintillation counting in a Beckman LS6500 system. The reaction reached equilibrium 5 min after addition of the substrate. Three independent determinations were in agreement within a 4% standard deviation from the mean.

Synthesis of [adenine-8-^{14}C]Adenosylcobalamin. To prepare adenosylcobalamin with a radiolabel in the adenosyl moiety, the following were first mixed together in dry form: 0.4 mg of Na_2ATP, 2.2 mg of hydroxocobalamin·HCl, 4.1 mg of $MgCl_2 \cdot 6H_2O$, and 10 mg of KBH_4. The mixture was brought into the anaerobic chamber along with [8-^{14}C]ATP and frozen adenosylcobalamin synthetase (CobA). When the enzyme solution had thawed, 250 μL each of 1 M Tris·HCl and [8-^{14}C]ATP (0.1 μCi/μL, 47 μCi/μmol) were mixed and the combination was brought to 2.5 mL with deaerated, deionized water. The dried ingredients were dissolved therein, and then 0.65 mg of CobA (60 μL) was added to begin the adenylation of cob(I)alamin. Care was taken during all subsequent steps to keep the solution shielded from light by keeping the reaction mixture in the dark room. The reaction mixture was incubated for 5 h at ambient temperature, at the end of which time the dark color of cob(I)alamin was still evident. An additional 10 mg of KBH_4 and 0.65 mg of CobA were added, with incubation continued for an additional 30 min. The reaction mixture was removed from the chamber, and air was bubbled through to oxidize any remaining cob(I)alamin. The precipitate was removed by passing the solution through a 0.45 μm syringe filter. The filtrate exhibited the cherry red color of adenosylcobalamin. Buffer and salts were removed by passing the solution through a 500 mg scale C_{18} Sep-Pak cartridge which had been activated by washing with methanol. After loading, the column was washed thoroughly with deionized water to remove buffer and salts, and then with a 0.02% TFA/10% methanol mixture to remove contaminating aquocobalamin. Adenosylcobalamin was eluted in a 0.02% TFA/40% methanol mixture, dried under vacuum, and redissolved in deionized water. The concentration of [*adenine*-8-^{14}C]adenosylcobalamin was determined by measuring the absorbance at 525 nm using an extinction coefficient of 8.0 mM^{-1} cm^{-1}. Purity was ascertained by liquid scintillation counting of fractions from a reversed phase C_{18} column using a 0 to 100% aqueous methanol gradient.

Synthesis of [5′,5′-$^2H_2$]Adenosylcobalamin. [5′,5′-$^2H_2$]-Adenosylcobalamin was synthesized enzymatically. The 0.5 mL solution containing 50 mM HEPES buffer (pH 7.5), 0.3 mM dGTP, 4 mM EDTA, 2 mM adenosylcobalamin, 0.2 mM NADPH, 30 mM DTT, and 0.05−0.1 mM ribonucleotide reductase (RTPR) in ^2H_2O was incubated at 37 °C for ~2 h. All subsequent steps were carried out in the dark room to keep the solution shielded from light. After the reaction was completed, the enzyme was removed by ultrafiltration, and the filtrate was loaded on a 500 mg scale C_{18} Sep-Pak cartridge that had been activated by washing with methanol. The column was washed thoroughly with deionized water to remove buffer and salts. Adenosylcobalamin was eluted in anhydrous ethanol, dried under vacuum, and redissolved in deionized water. HPLC using a C_{18} reversed phase column was applied for further purification of [5′,5′-$^2H_2$]adenosylcobalamin, using an elution gradient of 30 to 100% methanol in 0.02% TFA at a flow rate of 0.8 mL/min over the course of 20 min. The peak of adenosylcobalamin was pooled. The concentration of [5′,5′-$^2H_2$]adenosylcobalamin was determined by absorbance measurements at 341, 376, and 525 nm and calculated using extinction coefficients of 12.8, 11.0, and 8.0 mM^{-1} cm^{-1}, respectively. The purity of [5′,5′-$^2H_2$]-adenosylcobalamin was characterized by ^1H NMR (400 MHz) (*20*), LC−MS, and ESI-MS (data not shown).

Preparation of PLP-Free 5,6-LAM. 5,6-LAM substantially freed of PLP was prepared by dialyzing the protein against 20 mM triethanolamine·HCl buffer at pH 7.2 with 1 mM β-ME at 4 °C for 48 h. The PLP-freed enzyme typically retained less than 10% of the original activity, indicating that a small residue of PLP remained bound to the enzyme.

Recording UV−Visible Spectra under Anaerobic Conditions. In anaerobic experiments, the substrate (in the sidearm of an anaerobic cuvette) and the enzymatic mixtures (in the cuvette, 1.00 mL) were made anaerobic by repeated evacuation followed by purging with oxygen-free argon. The enzyme mixtures for spectrophotometry were made by mixing 5,6-LAM in 100 mM NH_4EPPS (pH 8.5), 5 mM β-ME, and various concentrations of PLP and adenosylcobalamin. After the blank spectra (deionized water as the reference) were obtained, inactivation was begun by addition of the substrate. Spectra from 190 to 800 nm were obtained once every 11 s for the first minute, and then the time interval was doubled between each succeeding spectrum.

Determination of the Inactivation Rate Constant ($k_{inactivation}$). Inactivation reactions were monitored by observing the generation of cob(III)alamin, the decomposition product of adenosylcobalamin, by UV−visible spectrophotometry (27 °C) or rapid scanning stopped-flow spectrophotometry (16 °C). The dramatic rise at 358 nm is the characteristic absorption for cob(III)alamin production, while the absorbance decreases at 466 nm. The distinctive double humps at 505 and 534 nm that develop with increasing incubation time

signal cob(III)alamin formation. The rate of inactivation was determined by plotting A_{358} versus time. The data were fitted well to the single-exponential growth function, $A_{358} = a(1 - e^{-kt}) + b$, in which k ($k_{inactivation}$) is the rate constant of inactivation.

HPLC-Monitored Production of 5′-Deoxyadenosine during Suicide Inactivation. An anaerobic inactivation solution was made consisting of 100 mM NH_4EPPS buffer at pH 8.5, 34.3 μM [*adenine*-8-^{14}C]adenosylcobalamin, 20 μM PLP, 34.3 μM 5,6-LAM, 5 mM NH_4PCA, 10 μM $FeCl_3$, and 0.1 unit of protocatechuate dioxygenase in a total volume of 294 μL. Turnover was initiated by the addition of 6 μL of 1 M D-lysine. Aliquots (30 μL) were taken at 0, 1, 2, 4, 8, 16, and 32 min and the reactions immediately quenched by the addition of 3 μL of 2 N $HClO_4$. The quenched samples were removed from the anaerobic chamber, centrifuged to remove protein, and neutralized with KOH. The 5′-deoxyadenosine was purified by HPLC using a C_{18} reversed phase column with a linear gradient from 0 to 100% methanol in 0.02% TFA at a flow rate of 0.9 mL/min over the course of 20 min. Peak elution was monitored at 260 nm. The peak identified as 5′-deoxyadenosine by co-injection of an authentic sample was pooled, and its ^{14}C content was quantified by liquid scintillation counting. Volumes corresponding to 5 min sections of the elution profile were also collected and analyzed to check for other ^{14}C-containing compounds that were present.

Rate of Suicide Inactivation. 5,6-LAM (40 μM) was incubated at 37 °C and pH 8.5 with 100 mM NH_4EPPS buffer, 5 mM β-ME, 100 μM adenosylcobalamin, 100 μM PLP, and 20 mM D-lysine in a total volume of 150 μL. Timed aliquots (5 μL) were mixed with excess PLP and adenosylcobalamin in 100 mM NH_4EPPS buffer and 5 mM β-ME (pH 8.5) and assayed for activity by incubation for 2 min with 100 mM D-[1-^{14}C]lysine. The inactivation and assays were carried out both aerobically and anaerobically to study the effect of dissolved oxygen on the inactivation. Anaerobicity of the enzyme and substrate was achieved by repeated evacuation followed by purging with oxygen-free argon, and assays were performed in the anaerobic chamber.

General Procedure for Substrate-Induced Suicide Inactivation. The incubation solutions contained 40 μM 5,6-LAM, 100 mM NH_4EPPS buffer (pH 8.5), 5 mM β-ME, and various concentrations of adenosylcobalamin and PLP in a total volume 150 μL. After preliminary incubation at 37 °C for 2−3 min, the reaction was initiated by the addition of various concentrations of D-lysine and allowed to proceed at 37 °C until the enzyme was fully inactivated. The inactivation was monitored by both activity assay and UV−visible spectra (with a 100 μL micro-quartz cuvette, monitoring A_{358}). After the inactivation was completed, the reaction was quenched with 5% TCA, and the precipitated protein was removed by centrifugation. The supernatant fluids were extracted with anhydrous ether (4 × 200 μL) to remove excess TCA, and the resulting solution was desalted by loading onto a C_{18} Sep-Pak cartridge, which had been activated. The salts, buffer, and polar molecules, such as lysine and PLP, were eluted by washing the column with deionized water. The less polar compounds, such as 5′-deoxyadenosine, were eluted with anhydrous ethanol.

Characterization of 5′-Deoxyadenosine as a Product of Suicide Inactivation. The incubation solutions contained 40 μM 5,6-LAM in 100 mM NH_4EPPS buffer (pH 8.5) with 40 μM adenosylcobalamin, 100 μM PLP, 5 mM β-ME, and 100 mM D-lysine or DL-lysine-d_8 in a total volume of 150 μL. After the general procedure described above had been carried out, the ethanol fraction was dried under vacuum and the resulting residues were dissolved in deionized water. The 5′-deoxyadenosine was purified by HPLC using a C_{18} reversed phase column with a linear gradient from 0 to 100% methanol in 0.02% TFA at a flow rate of 0.8 mL/min over the course of 30 min. Peak elution was monitored at 254 nm. The peak identified as 5′-deoxyadenosine by co-injection of an authentic sample was pooled, and the fraction was further characterized by LC−MS and ESI-MS.

Suicide Inactivation of 5,6-LAM in the Presence of [U-^{14}C]-PLP. PLP-freed 5,6-LAM (32 μM) was incubated with 5 mM β-ME, 100 mM NH_4EPPS buffer (pH 8.5), 80 μM adenosylcobalamin, 32 μM [U-^{14}C]PLP, and 100 mM D-lysine in a total volume of 200 μL. After inactivation, the enzyme was quenched with 5% TCA and the precipitated protein removed by centrifugation. The supernatant fluids were extracted with anhydrous ether to remove excess TCA, and HPLC analysis was carried out on Waters dimension C_{18} analytical column in a gradient of 0 to 80% methanol in 10 mM phosphate buffer (pH 6.5) at a flow rate of 1.0 mL/min over the course of 20 min. Peak elution was monitored at 254 nm. The amount of ^{14}C radioactivity of absorption peaks was counted on Beckman LS 6500 scintillation system.

Suicide Inactivation in ^3H_2O. In the inactivation solution, 0.047 μmol of 5,6-LAM was incubated at 37 °C with 150 μL of ^3H_2O (0.25 Ci) containing 50 mM K^+·EPPS buffer, 50 mM D-lysine, 2.5 mM β-ME, 0.094 μmol of PLP, and adenosylcobalamin. After the inactivation was completed, the solution was shell-frozen inside a round-bottom flask and the ^3H_2O sublimed into another flask. The residue was dissolved in deionized water and shell frozen and the water sublimed again. The process was repeated until only low levels of tritium were recovered in the sublimate. The residue was redissolved in 0.5 mL of water, and the protein was separated from other small molecule components by ultrafiltration. The protein and small molecule fractions were each radiochemically analyzed for tritium. The isolation of 5′-deoxyadenosine was described in the preceding section. Lysine and 2,5-DAH were derivatized by PITC (phenyl isothiocyanate), and the PITC-derivatized mixture of lysine and 2,5-DAH and PLP were purified by chromatography over a C-8 column (VYDAC) eluted with a gradient of 65% buffer A (0.05 M ammonium acetate) to 100% buffer B (0.1 M ammonium acetate in 44% H_2O, 46% CH_3CN, and 10% methanol) at pH 6.8. Peak elution was monitored at 254 nm. Volumes corresponding to 2−3 min sections of the elution profile were collected and checked for ^{3}H-containing compounds.

Purification of D-Lysine and 2,5-DAH after Suicide Inactivation. In incubation solutions similar to those for the general procedure, the concentrations of D-lysine or DL-lysine-d_8 were varied. At lower substrate concentrations (<20 mM), the inactivation was slowed. Following the general procedure, the aqueous fractions were dried by speed vacuum, and the resulting residues were dissolved in 0.5 mL of running buffer (40:40:15 $CH_3OH/CHCl_3$/12% NH_4OH). The resulting solutions were loaded onto short silica gel columns or silica Sep-Pak cartridges (650 mg), which had

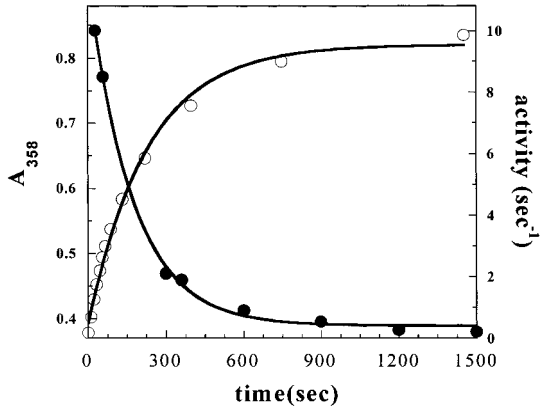


FIGURE 2: Cob(III)alamin production, as monitored by the increase in A_{358}, proceeds at the same rate as the loss of enzyme activity: (○) A_{358} and (●) residual enzymatic activity.

been pre-equilibrated by washing with running buffer. The mixtures of D-lysine and 2,5-DAH, which were detected by the acid ninhydrin reagent, were eluted with running buffer. The molecular mass of the mixture of D-lysine and 2,5-DAH was determined by ESI-MS.

RESULTS

The isomerization of D-lysine into 2,5-DAH is reversible. Because the migrating amino group is similarly bonded to carbon in the substrate and product, and the basicities of the amino groups are similar, the equilibrium constant is expected to be near unity. The actual value of K_{eq} for 2,5-DAH formation measured in this study at pH 8.5 and 37 °C was found to be 1.21 ± 0.05.

Cob(III)alamin Formation Concomitant with Suicide Inactivation. 5,6-LAM purified from *C. sticklandii* was reported to undergo inactivation in the absence of the activating enzyme E_2 and ATP (*1*). Substrate-dependent inactivation of the recombinant 5,6-LAM expressed in *E. coli* was also reported (*6*).

Suicide inactivation of recombinant 5,6-LAM is accompanied by cob(III)alamin production, as indicated by the increase in A_{358}, which proceeds at about the same rate as the loss of enzyme activity (Figure 2), with a rate constant of ~0.004 s^{-1}. The correlation suggests that the activity loss is related to the generation of cob(III)alamin. The formation of enzyme-bound cob(III)alamin associated with inactivation has been reported for other B_{12}-dependent enzymes (*11*, *22*). Cob(III)alamin formation presumably leads to inactivation of 5,6-LAM because adenosylcobalamin cannot be reformed, even if the 5′-deoxyadenosyl radical or lysyl-PLP adduct radical could somehow survive.

In the inactivation of 5,6-LAM, the cleavage of adenosylcobalamin to cob(III)alamin is accompanied by the formation of 5′-deoxyadenosine at the same rate, as shown in Figure 3. Moreover, the production of cob(III)alamin takes place at similar rates both aerobically and anaerobically, as shown in time-trace UV−visible spectra in Figure 4. The substrate-induced inactivation requires the presence of substrate (*6*), adenosylcobalamin, and PLP, and no cob(III)alamin is produced in the absence of either substrate or PLP (data not shown).

Two general mechanisms can be imagined to account for cob(III)alamin production. In one, Co−C bond cleavage of

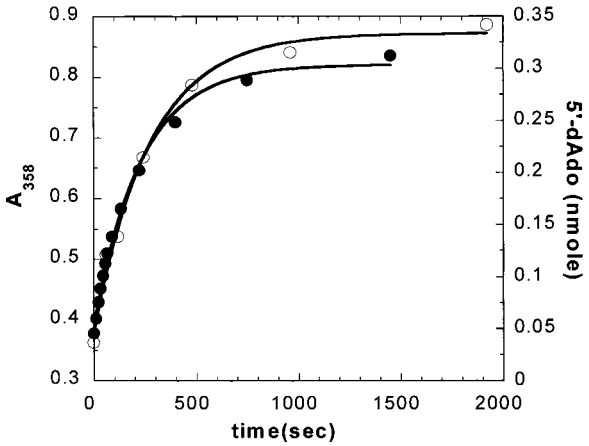


FIGURE 3: Formation of cob(III)alamin and 5′-deoxyadenosine by suicide inactivation of 5,6-LAM. The cleavage of adenosylcobalamin to cob(III)alamin (anaerobically) monitored by A_{358} during the substrate-induced inactivation (●) was accompanied by the formation of 5′-deoxyadenosine (O) at the same rate. The amount of radioactivity measured by scintillation counting of HPLC-purified 5′-deoxyadenosine from each time point is plotted vs inactivation time.

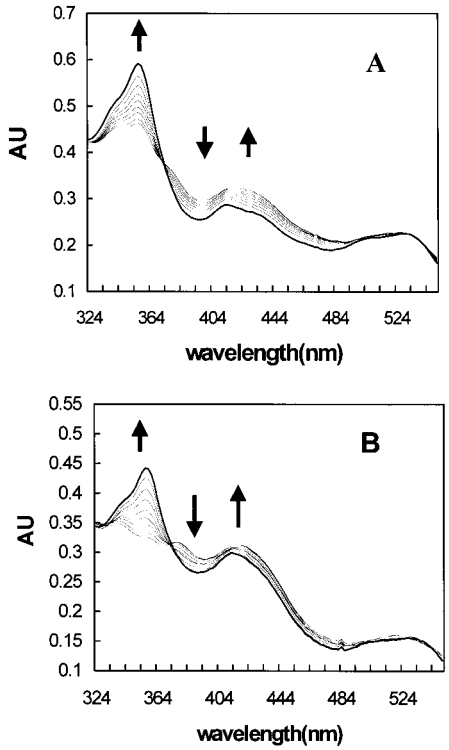


FIGURE 4: Anaerobic and aerobic production of cob(III)alamin in suicide inactivation of 5,6-LAM. Deionized H_2O was used as a blank reference. The solution contained 13.5 μM 5,6-LAM, 20 μM PLP, 15 μM adenosylcobalamin, 5 mM β-ME, and 100 mM NH_4-EPPS in a total volume of 980 μL. The reaction was started by the addition of D-lysine (20 mM), and spectra were recorded 1.1, 12.4, 23.2, 34.3, 45.3, 56.4, 67.3, 89.4, 133, 221, 397, 749, and 1450 s after initiating inactivation. Only representative spectra are shown.

adenosylcobalamin occurs heterolytically in a side reaction that is irrelevant to amino mutation. Two types of heterolytic cleavage of adenosylcobalamin under acidic conditions have been described (*24*, *25*). However, in both cases, the nucleoside products were adenine and 2,3-dihydroxy-4-pentenal. Our observation of 5′-deoxyadenosine as the major nucleoside product of the inactivation rules out heterolytic bond cleavage to generate cob(III)alamin.

Table 1: Kinetic Parameters of D-Lysine, L-Lysine, and β-Lysine as Substrates and Suicide Inactivators

substrate	K_M (mM)	V_{max} (min^{-1})	k_{inact} (min^{-1})[a]	K_i (mM)	inhibition pattern
D-lysine	20.0 ± 1.0	800.7 ± 44.4	0.702 ± 0.042		
L-lysine	20.3 ± 5.6	107.7 ± 7.3	0.570 ± 0.042	23.0 ± 2.2	comp[b]
β-lysine	ND[c]	ND[c]	>18[d]	0.0044 ± 0.0007	comp[b]

[a] k_{inact} ($k_{inactivation}$) was calculated from the data of the UV−visible time course. [b] Competitive inhibition. [c] Not determined. [d] Estimated from the results of stopped-flow spectrophotometry.

Another possible mechanism entails a side reaction of a putative radical intermediate, which is quenched by electron transfer from cob(II)alamin followed by protonation. Alternatively, a radical intermediate may abstract a hydrogen atom from a nearby group, such as an amino acid side chain in the active site. Electron transfer from cob(II)alamin followed by protonation would quench the resulting protein radical. In either case, the catalytic cycle would be permanently interrupted by the impossibility of regenerating adenosylcobalamin.

Absence of Deuterium Kinetic Isotope Effects in Suicide Inactivation. To clarify certain aspects of the mechanism of suicide inactivation, studies of kinetic isotope effects were undertaken. The inactivation rates were very similar when inactivation was carried out with unlabeled adenosylcobalamin versus [5′,5′-$^2H_2$]adenosylcobalamin, with D-lysine versus DL-lysine-d_8, or in H_2O versus ^2H_2O (data not shown). Therefore, in the absence of a deuterium kinetic isotope effect, it is very unlikely that hydrogen transfer limits the inactivation rate.

Irreversibility of Substrate-Induced Inactivation. As in the cases of other reported adenosylcobalamin-dependent enzymes, such as dioldehydrase (*11*), we found that enzyme-bound cob(III)alamin remained tightly bound to 5,6-LAM and could not be exchanged with free adenosylcobalamin (data not shown). The irreversibility of inactivation also confirmed the results of activity assays, in which the assay mixtures contained excess adenosylcobalamin and PLP. In addition, the rate of inactivation was not increased by the presence of >1 equiv of adenosylcobalamin relative to 5,6-LAM.

Rates of Inactivation by D-, L-, and β-Lysine. 5,6-LAM has been reported to display dual substrate specificity, and rapid inactivation by both substrates was reported in the absence of component E_2 (*1*). β-Lysine was also reported to be a better substrate than D-lysine for the enzyme, and it also appeared to inactivate the enzyme faster. In rapid scan stopped-flow spectrophotometric experiments under aerobic conditions, we were able to calculate the rate constant for inactivation by β-lysine at 16 °C (lower incubation temperature) (Table 1 and Figure 5). Inactivation by β-lysine was found to be at least 25 times faster than by D-lysine.

Although 5,6-LAM has been reported not to accept L-lysine as a substrate, our spectrophotometric experiments showed that L-lysine led to the production of cob(III)alamin at a rate similar to that brought about by D-lysine. Inactivation by L-lysine was not due to contamination by D-lysine, as indicated by analysis of the purity of L-lysine by chiral column chromatography (CHIROBIOTIC T, Astec). In addition, measurements of the initial reaction rate, using the radiochemical assay and various concentrations of L-[U-^{14}C]-lysine, indicated that L-lysine also served as a substrate, although at a lower rate than with D-lysine. The steady-state kinetic parameters for D- and L-lysine are given in Table 1.

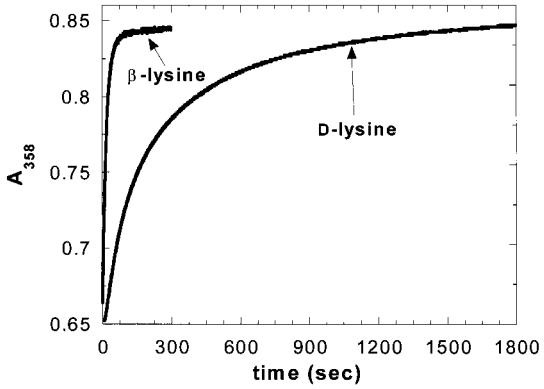


FIGURE 5: Cleavage of adenosylcobalamin to cob(III)alamin by D-lysine and β-lysine at the active site of 5,6-LAM. Cob(III)alamin production under aerobic conditions induced by D-lysine (20 mM) and β-lysine (20 mM), as monitored by A_{358} in stopped-flow spectrophotometry. Deionized H_2O was used as a blank reference. The reaction components and concentrations are as described in the legend of Figure 4.

In the inhibition studies, both β-lysine and L-lysine behave as competitive inhibitors versus D-lysine, which is consistent with the expectation that the enzyme should have only one active site for D-, L-, and β-lysine instead of two or multiple active sites for different substrates (*1*). Among these substrates, it appears that β-lysine may fit best into the active site pocket, since the K_i for β-lysine has been estimated to be $\sim$4 μM, which is much lower than the K_m values for D-lysine and L-lysine, under both aerobic and anaerobic conditions. Values for K_m and V_{max} for β-lysine were not determined due to the fast substrate-induced inactivation.

The Putative 5′-Deoxyadenosyl Radical Is Not Involved in the Substrate-Induced Inactivation. Because oxygen scavenging of an intermediate cannot explain suicide inactivation (Figure 4), we considered whether electron transfer from cob(II)alamin to a putative free radical intermediate in the catalytic cycle could explain inactivation and cob(III)-alamin formation. Four possibilities are outlined in Figure 6, which describes the course of inactivation if any one of the four radicals in the hypothetical mechanism in Figure 1 should be quenched by electron transfer followed by protonation. We sought to determine whether a side reaction of one of the radical intermediate accompanied the inactivation.

The most common feature of adenosylcobalamin-dependent reactions is initiation of radical formation by enzyme-induced homolytic cleavage of the Co−C bond to generate cob(II)alamin and the 5′-deoxyadenosyl radical. In the preliminary inactivation studies, we found that the generation of cob(III)alamin was accompanied by the formation of 5′-deoxyadenosine at the same rate (Figure 3). Therefore, a side reaction that oxidizes cob(II)alamin and quenches the 5′-deoxyadenosyl radical to 5′-deoxyadenosine would lead to suicide inactivation. For example, if electron transfer from cob(II)alamin to the putative 5′-deoxyadenosyl radical should

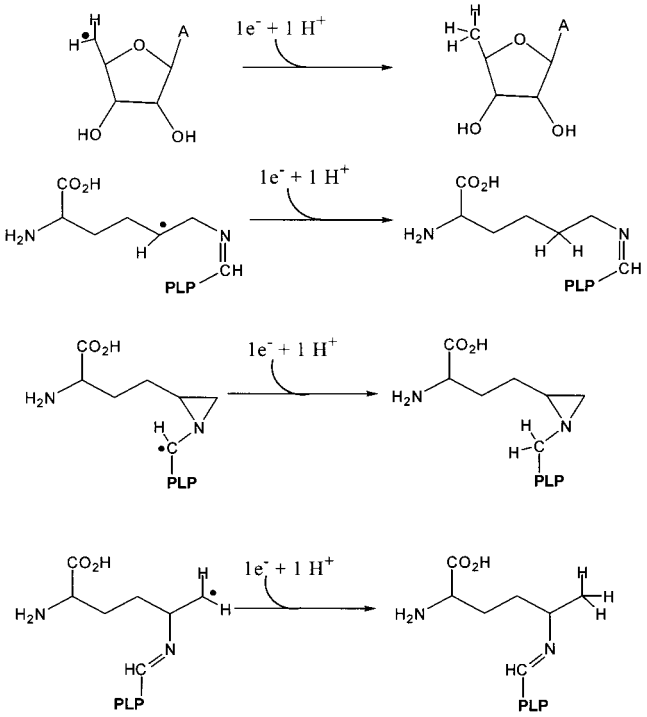


FIGURE 6: Hypothetical schemes for the substrate-induced inactivation process observed in 5,6-LAM. Illustrated here are the overall courses of hypothetical suicide inactivation in the event that a radical intermediate accepts an electron from cob(II)alamin. The radicals are the four radical intermediates in the hypothetical catalytic mechanism depicted in Figure 1.

Table 2: Origin of Hydrogen in 5′-Deoxyadenosine after Suicide Inactivation under Different Reaction Conditions[a]

substrate	solvent	*m*/*z* of 5′-deoxyadenosine[b]
D-lysine	H_2O	252
D-lysine	^2H_2O	252
DL-lysine-d_8	H_2O	255
L-lysine-d_8	H_2O	255

[a] The reaction condition is described in Materials and Methods. [b] The mass was measured in the positive ion mode by LC−MS and ESI-MS.

take place, followed by protonation of the anion, cob(III)alamin would be formed and regeneration of adenosylcobalamin would be blocked. Alternatively, another adventitious enzymatic oxidation may lead to cob(III)alamin production from cob(II)alamin, stranding 5′-deoxyadenosine in its intermediate state. In this latter case, the putative 5′-deoxyadenosyl radical would not participate in the inactivation.

Two experiments tested the above hypotheses. Stoichiometrically equivalent amounts of adenosylcobalamin and 5,6-LAM were combined, and the solution was incubated with either DL-lysine-d_8 in H_2O or D-lysine in ^2H_2O until inactivation was nearly complete. The resulting 5′-deoxyadenosine was purified, and the samples were characterized by LC−MS or ESI-MS (Table 2, in the positive ion mode). When DL-lysine-d_8 was the substrate, trideutero-5′-deoxyadenosine (*m*/*e* 255) was isolated. On the other hand, unlabeled 5′-deoxyadenosine (*m*/*e* 252) was produced when the enzyme was incubated with D-lysine in ^2H_2O. Taken together, the results prove that in substrate-induced inactivation the putative 5′-deoxyadenosyl radical acquires hydrogen exclusively from either the substrate or product. Therefore, because solvent hydrogen was not incorporated into 5′-deoxyadenosine, no evidence indicating participation of the 5′-deoxyadenosyl radical in a side reaction was obtained. In particular, it is most unlikely that electron transfer from cob(II)alamin to the 5′-deoxyadenosyl radical could have taken place. If it had, either the resulting carbanion would have decomposed to adenine and 2,3-dihydroxy-4-pentenal, which did not take place, or it would have been quenched by solvent protonation to 5′-deoxyadenosine, which also did not occur.

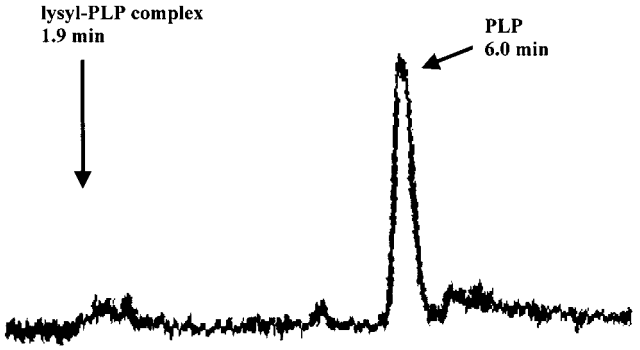


FIGURE 7: Recovery of PLP after suicide inactivation of 5,6-LAM in the presence of [U-^{14}C]PLP. PLP-freed 5,6-LAM was incubated in [U-^{14}C]PLP under substrate-induced inactivation conditions. After the reaction had been quenched, PLP was subjected to chromatography on a reversed phase C_{18} HPLC column (see Materials and Methods for details). Fractions were monitored at 254 nm, and only representative chromatograms were shown. Every peak was pooled and analyzed radiochemically. Most of the activity was at a 6.00 min fraction coeluting with PLP.

The Putative Aziridylcarbinyl Radical Is Not Quenched in Suicide Inactivation. If the proposed aziridylcarbinyl radical intermediate accepts an electron from cob(II)alamin, proton transfer from the solvent or a nearby solvent exchangeable site would likely quench the resulting C4′ anion. Alternatively, the aziridylcarbinyl radical may acquire hydrogen from an unexpected source. In either case, the PLP−lysyl complex would be produced. To test these hypotheses, we incubated the PLP-freed 5,6-LAM with [U-^{14}C]PLP, and characterized all radioactive fractions after the inactivation experiment was completed. From the HPLC analysis (Figure 7), the major absorption peaks were collected and analyzed radiochemically. After co-injection with authentic PLP and the chemically synthesized PLP−lysyl adduct, we observed a quantitative recovery of [U-^{14}C]PLP (t_R = 6.0 min) and no [U-^{14}C]PLP−lysyl adduct (t_R = 1.9 min) after inactivation. Therefore, the direct protonation of the C4′ anion, the third mechanism in Figure 6, is ruled out, as well as any misdirected adventitious hydrogen transfer to the intermediate **3** in Figure 1.

Solvent Hydrogen Incorporation into the Substrate/Product Mixture. According to the second and fourth mechanisms in Figure 6, electron transfer from cob(II)alamin to the substrate- and/or product-related side chain radicals followed by protonation would lead to cob(III)alamin formation and the incorporation of solvent protons into the substrate and/or product. The incorporation of ^2H from ^2H_2O can be monitored by ESI-MS analysis, and the level of tritium incorporation from ^3H_2O can be determined by liquid scintillation counting. When the enzyme was incubated with DL-lysine-d_8 (*m*/*z* 155, in the positive ion mode), the lysine and product mixtures were re-isolated and purified after the enzyme was fully inactivated. The *m*/*z* 154 peak corresponding to DL-lysine-d_7 was of considerable interest (Table 3).

Table 3: *m/z* Ratios of Lysine/2,5-DAH Mixtures after Suicide Inactivation (Incorporation of Solvent Hydrogen)

[DL-lysine-d_8][a] (mM)	[enzyme] (μM)	cofactor	solvent	*m/z* 154/155 × 100[b] (%)
	0	−	H_2O	3.43 ± 0.13
20	18	[5′,5′-H_2]adenosylcobalamin	H_2O	4.17 ± 0.04
20	18	[5′,5′-$^2H_2$]adenosylcobalamin	H_2O	3.70 ± 0.05
4.0	100	[5′,5′-$^2H_2$]adenosylcobalamin	H_2O	6.77 ± 0.03
4.0	100	[5′,5′-$^2H_2$]adenosylcobalamin	^2H_2O	4.13 ± 0.08

[a] DL-Lysine-d_8 is DL-[3,3,4,4,5,5,6,6-$^2H_8$]lysine. [b] The peak areas of mass spectra were measured by ESI-MS, in the positive ion mode.

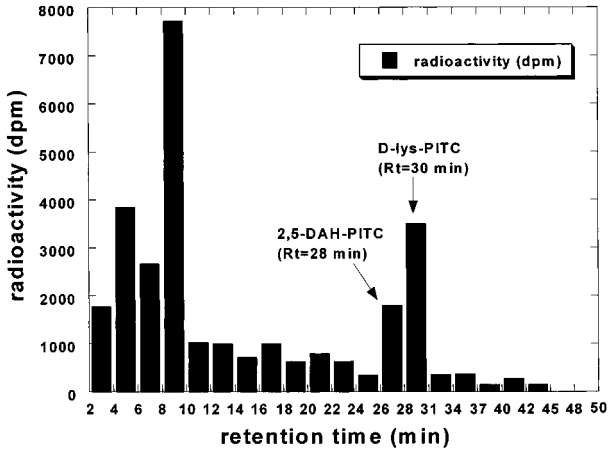


FIGURE 8: Observation of tritium in D-lysine and 2,5-DAH after suicide inactivation of 5,6-LAM in ^3H_2O. After suicide inactivation in ^3H_2O and removal of excess tritium, the products were separated chromatographically. Shown are the relevant sections of the chromatogram for the separation of the PITC derivatives of D-lysine and 2,5-DAH. Tritium is associated with both derivatives.

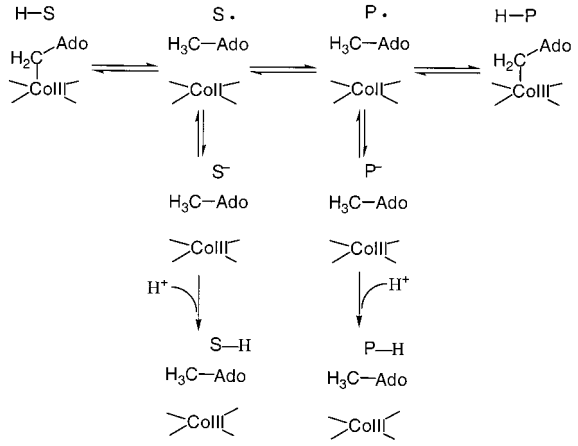
The 154/155 ratio for DL-lysine-d_8 represented slightly incomplete deuteration of the molecule. If in an inactivation experiment a proton should be incorporated into the substrate or product, the 154/155 ratio should increase. Line 2 in Table 3 indicates that it does increase in samples obtained from suicide inactivation reactions in water. Line 3 of Table 3 indicates that the ratio declines somewhat when [5′,5′-$^2H_2$]-adenosylcobalamin is substituted for adenosylcobalamin, as it should by increasing the deuterium in the hydrogen pool. Line 4 of Table 3 shows that decreasing the ratio of DL-lysine-d_8 to enzyme dramatically increases the 154/155 ratio, as it should if protonation from water increases the size of the pool of DL-lysine-d_7 at the expense of DL-lysine-d_8. Repeating the same experiment in D_2O dramatically decreased the 154/155 ratio, confirming the incorporation of solvent protons into the mixture of D-lysine and 2,5-DAH.

In an analogous experiment with ^3H_2O, the PITC-derivatized substrate (t_R = 30 min) and product (t_R = 28 min) were purified and both found to contain detectable amounts of tritium after the inactivation (Figure 8). The protein, 5′-deoxyadenosine, and PLP were not labeled with tritium in this experiment. Taken together, our results showed the incorporation of solvent hydrogen into the mixture of D-lysine and 2,5-DAH, indicating that substrate and product intermediates were involved in the generation of cob(III)alamin and suicide inactivation.

DISCUSSION

Suicide Inactivation by Electron Transfer. The results of our investigation into the suicide inactivation of 5,6-LAM show that the process leads to the cleavage of adenosylcobalamin into cob(III)alamin and 5′-deoxyadenosine, that this process does not involve dioxygen, and that solvent hydrogen is stably incorporated into the pools of D-lysine and 2,5-DAH. Solvent hydrogen is not incorporated into 5′-deoxyadenosine or PLP, and PLP is not trapped into the form of a complex with the substrate or product.

Scheme 1



The simplest inactivation mechanism that is consistent with these facts is one based on the hypothetical catalytic mechanism in Figure 1. The most straightforward and obvious possibilities are the second and fourth inactivation processes outlined in Figure 6, whereby the putative cob(II)alamin adventitiously transfers an electron to a substrate- or product-based radical intermediate in a side reaction. This would lead to a transient carbanionic species that would be irreversibly quenched by protonation. The ultimate source of quenching protons is the solvent, although it may not be the solvent itself that donates a proton at the active site. More likely, an amino acid side chain or a peptide N−H group would serve as the immediate proton donor in quenching a transient carbanionic species.

The overall course of the process can be described by Scheme 1. The upper line describes a minimal mechanism for the role of adenosylcobalamin in the interconversion of the substrate into the product by way of substrate and product radicals and cob(II)alamin as intermediates. Occasional electron transfer from cob(II)alamin to the substrate or product leads to cob(III)alamin and transient carbanionic species, which are irreversibly quenched by protonation. The results from suicide inactivation in ^3H_2O, shown in Figure 8, indicate that tritium is incorporated into both D-lysine and 2,5-DAH, suggesting the participation of both the substrate and product radicals in electron transfer. The results implicate at least the substrate or the product in electron transfer, but they are not clear enough to implicate both of them definitively.

The question of the mechanism of electron transfer is not resolved by the experiments described herein. The simplest

possible mechanism is direct electron transfer from cob(II)alamin to a substrate or product radical, as implied in Scheme 1. The radicals are likely to be near enough to cob(II)alamin at the active site to allow this mechanism, and in the absence of other evidence, the simplest mechanism must be regarded as the most likely. However, consideration of the hypothetical overall catalytic mechanism in Figure 1 suggests the possibility that a more complex mechanism could be at work. The central intermediate in Figure 1 is radical **3**, the aziridylcarbinyl radical. It is likely to be as accessible to electron transfer from cob(II)alamin as any other radical, and because of delocalization of an incipient carbanion into the pyridine ring, it may be a more favorable electron acceptor than the other radicals. If the 4′-carbanion resulting from electron transfer to radical **3** were to be inaccessible to protonation, it might undergo ring opening to the substrate or/and product carbanions, which could then be quenched by protonation. This more complex mechanism would also account for the results presented here.

Even more complex electron transfer mechanisms could be written. In such mechanisms, an enzymatic functional group such as a cysteinyl SH or another amino acid side chain with a heteroatomic functional group, or even an amide N−H group, would participate in electron transfer and misdirected hydrogen transfers with the substrate and product radicals. In the absence of evidence for such processes, simpler mechanisms should be regarded as more likely to be in operation.[2]

While our studies suggest that inactivation proceeds by electron transfer from cob(II)alamin to a recipient to form cob(III)alamin, we never actually observe cob(II)alamin as an intermediate. This is presumably because cob(II)alamin cannot accumulate to a detectable concentration in the steady state. Nevertheless, evidence for the suicide inactivation mechanisms in Figure 6 and Scheme 1 is not based on the experimental observation of cob(II)alamin. In this connection, it is interesting that an analogous suicide inactivation is observed in the reaction of dioldehydrase with 3′,4′-anhydroadenosylcobalamin. This analogue of adenosylcobalamin serves as a coenzyme at a low rate, but in the absence of a substrate, it is cleaved at the active site. The initial cleavage leads to cob(II)alamin, which is then transformed into cob(III)alamin in an O_2-independent process.[3] The nucleoside product has been identified as 5′-deoxy-3′,4′-anhydroadenosine. Available evidence implicates electron transfer from cob(II)alamin to the 5′-deoxy-3′,4′-anhydroadenosyl radical followed by protonation of the resulting carbanion.

As mentioned in preceding sections, substrate-induced inactivation is also observed in other adenosylcobalamin-dependent enzymes (*11*, *22*). Apparently, these enzymes, which carry out catalysis by a radical mechanism, have a tendency to undergo mechanism-based inactivation. Unlike methylmalonyl-CoA mutase, 5,6-LAM is inactivated anaerobically during turnover, which means a process other than oxygen scavenging leads to suicide inactivation. In this report, we found that solvent hydrogen incorporates into the substrate/product mixture, and this result supports the idea that substrate and/or product radicals are involved in the inactivation through acquisition of an electron from cob(II)alamin either directly or indirectly.

Prevention or Correction of Suicide Inactivation in the Cell. The suicide inactivation described here would interfere with the function of 5,6-LAM in cells. A mechanism should exist to maintain the intracellular activity. A protein termed E_2 has been described that activates 5,6-LAM and appears to be ATP-dependent (*1*, *5*). The exact function of E_2 is not known with certainty, although it has been described as an adenosylcobalamin synthetase. Synthetase activity would presumably allow for the replenishment of adenosylcobalamin from the cob(III)alamin generated at the active site of 5,6-LAM by suicide inactivation, and this would explain the participation of ATP. Other modes of action for E_2 are possible, however. For example, E_2 might bind to 5,6-LAM and prevent suicide inactivation. Alternatively, E_2 may facilitate the exchange of cob(III)alamin at the active site with free adenosylcobalamin in solution. Research in progress seeks to elucidate the role of E_2 and the mechanism by which it activates 5,6-LAM.

[2] The presence of β-ME in the buffer cannot contribute to this process because its exclusion does not affect the course or timing of the suicide inactivation.

[3] O. Th. Magnusson and P. A. Frey, manuscript in preparation.

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