Identification of a Rearranged-Substrate, Product Radical Intermediate and the Contribution of a Product Radical Trap in Vitamin B_{12} Coenzyme-Dependent Ethanolamine Deaminase Catalysis

Kurt Warncke,* Jennifer C. Schmidt, and Shyue-Chu Ke

Contribution from the Department of Physics, Emory University, Atlanta, Georgia 30322

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Abstract: The radical intermediate present during steady-state turnover of substrate aminoethanol by ethanolamine deaminase from *Salmonella typhimurium* has been characterized by using X-band electron paramagnetic resonance (EPR) spectroscopy. The radical intermediate was prepared by cryotrapping enzyme, aminoethanol substrate, and vitamin B_{12} coenzyme (adenosylcob(III)alamin) immediately following mixing. Natural abundance, 1,1,2,2-$^2H_4$-, 2-^{13}C-, and 1,2-$^{13}C_2$-aminoethanol were used as substrates. The EPR spectrum obtained for natural abundance aminoethanol shows a broad feature at approximately $g = 2.3$ that arises from Co^{II} in cob(II)alamin, and a feature from an organic radical that has an absorption maximum at $g = 2.02$ and a line width of 10.8 mT. The EPR line shape is characteristic of a relatively weakly electron spin-coupled Co^{II}-organic radical system. The EPR line shapes for the ^2H- and ^{13}C-labeled substrates were narrowed and broadened by 0.7 and 2.4 mT, respectively, demonstrating that the radical is substrate-based. The comparable line widths of the 2-^{13}C- and 1,2-^{13}C-labeled radicals show that the unpaired spin density is localized primarily at the C_2 carbon atom. This identifies the radical intermediate as a rearranged substrate radical, or product radical. The results are consistent with either the 1-aminoethanol-2-yl radical or the ethanal-2-yl radical, which have been proposed as intermediates in, respectively, the amine migration and amine elimination mechanisms of rearrangement. A qualitative reaction free energy profile for the Co^{II}-radical pair intermediate states on the enzyme is constructed, based on the EPR results and previous isotope exchange and kinetic isotope effect studies. The results and analysis reveal that a product radical trap strategy contributes to the stabilization of the radical pair state, which enhances catalytic performance of ethanolamine deaminase.

Introduction

The family of vitamin B_{12} coenzyme-dependent enzymes catalyze the radical-mediated cleavage of unactivated C−H bonds and associated 1,2-*cross*-migrations of hydrogen and alkyl, carbonyl, hydroxyl, or amino groups.[1−5] The cycle of vitamin B_{12}-coenzyme-mediated enzyme catalysis is depicted in Figure 1 for the bacterial ethanolamine deaminase,[1,3,6,9] which catalyzes the deamination of aminoethanol[9] to the products ammonia and ethanal (acetaldehyde).[10] In the first step of the proposed reaction sequence, the cobalt−carbon bond in enzyme-bound vitamin B_{12} coenzyme [adenosylcob(III)alamin] is cleaved homolytically to form $S = 1/2$, low spin Co^{II} in cob(II)alamin and a putative 5′-deoxyadenosyl radical.[12] The 5′-deoxyadenosyl radical has been proposed[1,3,6] to abstract a hydrogen atom from substrate aminoethanol **1** in the first hydrogen transfer step, HT1, generating the 2-aminoethanol-1-yl substrate radical **2**. The additional participation of a protein-associated radical intermediate between the deoxyadenosyl and substrate-derived radicals has been proposed.[7] The electron-deficient substrate radical **2** is thus activated for rearrangement to a product radical **3**. In a second hydrogen transfer step, HT2, the product radical abstracts a hydrogen atom from the 5′-methyl group of deoxyadenosine to form the diamagnetic product **4**, which dissociates from the enzyme. It is thought that the 5′-deoxyadenosyl radical and Co^{II} in cob(II)alamin then recombine to regenerate the intact coenzyme prior to the next turnover.

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(9) This enzyme was originally named "ethanolamine deaminase"[10,11] (E.C. 4.3.1.7), and was later also called "ethanolamine ammonia-lyase" by some groups, in particular, in the post-1970 portion of the extensive work from the laboratory of B. M. Babior. In contrast to the use of the historical common name for the enzyme, we follow the IUPAC conventions in the nomenclature for substrates and their derivatives. This avoids the somewhat confusing dual use of common and IUPAC nomenclature in the early literature. For example, we refer to the natural substrate as 2-aminoethanol, or simply aminoethanol, rather than ethanolamine.
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(12) The failure to directly detect the 5′-deoxyadenosyl radical by rapid reaction quench methods,[13] and a substrate deuterium isotope effect on cobalt−carbon bond cleavage in methylmalonyl-CoA mutase,[14] has led to the suggestion that hydrogen abstraction from substrate may proceed without formation of the 5′-deoxyadenosyl radical as a distinct chemical intermediate.[13,14] A large substrate deuterium isotope effect on the rate of cobalt−carbon bond cleavage has also been observed in glutamate mutase, but a low concentration, high energy 5′-deoxyadenosyl radical intermediate is favored as an explanation.[15] Magnetic field effects on the ethanolamine deaminase reaction indicate the formation of an intermediate deoxyadenosyl-Co^{II} radical pair in this system.[16,17]

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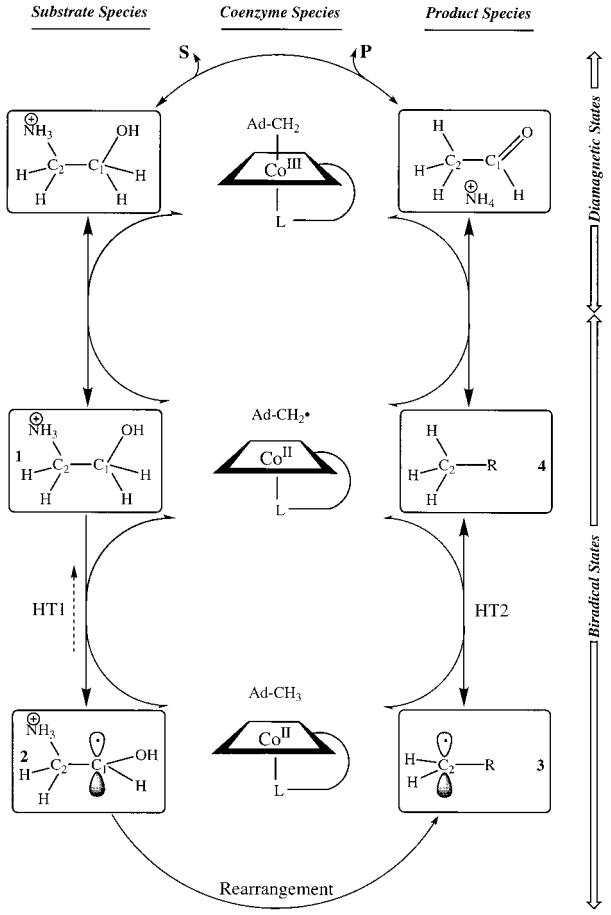

Figure 1. Depiction of the cycle of vitamin B_{12} coenzyme-dependent enzyme catalysis in ethanolamine deaminase, showing bound states of the vitamin B_{12} coenzyme, substrate, and product species.[3,6] The forward direction of the reaction proceeds in the counterclockwise sense. The intermediates and steps represent a minimal mechanism. For example, involvement of a protein radical intermediate in steps HT1 and HT2 has been proposed.[7] Substrate-derived species in the radical pair states are designated **1** (bound substrate), **2** (substrate radical), **3** (product radical), and **4** (bound products). An R-group represents the C_1 center in **3** and **4**, because the state of substitution at C_1, which involves the fate of the amine/ammonium group, is unknown.[3] The corrin ring of the coenzyme is represented as a foreshortened square, with attached nucleotide loop and dimethylbenzimidazole α-axial ligand, L. The dimethylbenzimidazole ligand remains bound to the coenzyme upon binding and during catalysis in ethanolamine deaminase.[8] The 5′-deoxyadenosyl β-axial ligand is represented as Ad-CH_2- in the intact coenzyme, and as Ad-CH_2• (5′-deoxyadenosyl radical) or Ad-CH_3 (5′-deoxyadenosine) following cobalt−carbon bond cleavage. **S** and **P** represent substrate aminoethanol and products ethanal and ammonia, respectively. HT1 and HT2 are the first and second hydrogen transfer reactions, respectively, in the forward direction. The reaction sequence and characterization of the intermediates is described in the text.

Two principal challenges to understanding the molecular basis of catalysis by ethanolamine deaminase and other adenosylcobalamin-dependent enzymes are the determination of the factors that promote radical pair stabilization and the elucidation of the radical rearrangement mechanism. The effective rate of cleavage of the cobalt−carbon bond that generates the initial radical pair is accelerated by approximately 10^{12}-fold when the coenzyme is bound to the enzyme versus free in solution.[13] The factors that counter geminate radical pair recombination appear to act on the subnanosecond time scale,[19,20] which is beyond the deadtime limitations of rapid freeze-quench and stopped-flow experiments.[13−15,17,21−24] However, two observations suggest that "late" micro- to millisecond phases of the reaction that involve substrate-derived radical species participate in the radical pair stabilization process. First, cobalt−carbon bond cleavage depends obligatorily on substrate binding in all adenosylcobalamin-dependent enzymes, with the exception of ribonucleotide triphosphate reductase, which can cleave the bond upon binding a nucleotide triphosphate activator.[5] Second, the optically detected bond cleavage kinetics depend on the rate of the first hydrogen atom abstraction reaction from substrate in the methylmalonyl-CoA mutase[14] and glutamate mutase[15] systems.[25] This has led to the suggestion that formation of the substrate radical acts to effectively trap the radical pair in glutamate mutase.[15] In ethanolamine deaminase, the energetic and underlying molecular structure contributions to radical pair stabilization remain unknown, with the exception of the contribution of enhanced separation distance between Co^{II} and the substrate-derived radicals.[26,27] Likewise, the mechanism of the terminal step in the radical pair-associated reactions, the radical rearrangement, is not known for ethanolamine deaminase.[3]

To determine the mechanisms of the radical pair stabilization and radical rearrangement processes in ethanolamine deaminase and other vitamin B_{12} coenzyme-dependent enzymes, radical catalytic intermediate states must by prepared and their structures characterized. Direct examination of radical intermediates by using techniques of electron paramagnetic resonance (EPR) spectroscopy in tandem with specific isotope labeling on the substrate can reveal two key types of information bearing on the enzyme mechanism. First, the isotope position-dependence of the EPR line shape can reveal the identity of the radical through changes in the electron−nuclear hyperfine interactions. Second, the steady-state or equilibrium accumulation of EPR-distinguishable radical states reflects the free energy surface for the reaction and, in concert with isotope exchange and kinetic results, can indicate the energetic contributions to catalysis. EPR spectra of coenzyme B_{12} systems have been obtained following rapid freeze-quench of enzyme−coenzyme−substrate mixtures or by cryotrapping under turnover conditions. Two general types of biradical EPR line shapes are observed that are distinguished by the strength of the electron spin−spin coupling between Co^{II}

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(25) The absence of a substrate deuterium isotope effect on the rate of cob(II)alamin formation in ethanolamine deaminase has been reported.[17] However, in contrast to the other stopped-flow studies in which holoenzyme was reacted with substrates,[14,15] this result was obtained without preequilibration of enzyme and coenzyme.[17] Thus, the pronounced lag in the rise to the steady state,[11,23] proposed to be caused by a change in protein conformation,[23] may have obscured a substrate kinetic deuterium isotope effect.
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in cob(II)alamin and the substrate-derived organic radical.[28] In one type, which is common to the Class II vitamin B_{12} coenzyme-dependent enzymes that mediate heteroatom eliminations, the coupling is sufficiently weak that the dominant $g_\perp$ resonance of Co^{II} ($g = 2.2-2.3$)[29] and the organic radical resonance centered around $g = 2.0$ are spectrally separated.[26,30,31] Babior and co-workers compared the $g = 2.0$ EPR line shapes in ethanolamine deaminase generated with selectively ^2H- and ^{13}C-labeled 2-aminopropanol, a substrate analogue, to show that the radical was the initially formed, *unrearranged* substrate radical, represented by **2** in Figure 1.[21,32] Remarkably, this is the only study in which a weakly coupled organic radical has been previously directly identified. EPR signals generated during turnover of *Clostridium* sp. ethanolamine deaminase on the natural substrate, aminoethanol, of varying line shape were reported, but their origin was not characterized.[35,36] In diol dehydrase,[37] 1,1-^2H-labeling of the natural substrate, 1,2-propanediol, caused no detectable change in the $g = 2.0$ radical line shape, whereas the line shape of the radical formed from the enzyme inactivator chloroacetaldehyde was influenced by ^2H- and ^{13}C-labeling of chloroacetaldehyde.[38] The set of labeled compounds was not extensive enough to venture structure assignments.[38] The EPR line shape of the biradical in glycerol dehydrase was not addressed with isotopically labeled substrates.[39] A $g = 2.0$ doublet radical line shape formed on the minutes time scale in the ribonucleotide triphosphate reductase from *Lactobacillus leichmannii* was not detectably influenced by ^2H_2O exchange, ^2H-labeled nucleotide triphospate substrate, and 5′-$^2H_2$-deoxyadenosine.[40,41]

The second general type of biradical line shape observed in B_{12} coenzyme-dependent enzymes arises from relatively strong electron spin−spin coupling and is characterized by a single dominant feature displaying cobalt hypefine coupling and a zero-crossing *g*-value approximately midway between the Co^{II} $g_\perp$ and organic radical *g*-values.[24] The strongly coupled biradical EPR line shape is characteristic of the Class I vitamin B_{12} coenzyme-dependent enzymes that catalyze carbon skeleton rearrangements, and has been observed in glutamate mutase,[42] methylene glutarate mutase,[43] and methylmalonyl-CoA mutase.[44,45] EPR spectroscopy and ^2H- and ^{13}C-labeled glutamate substrates identified the contribution of the unrearranged, 4-glutamyl substrate radical to the biradical in glutamate mutase.[46] The methylene glutarate mutase radical has not been addressed with isotopically labeled substrates,[43] while for methylmalonyl-CoA mutase, labeling of substrate with ^{13}C or ^2H caused no significant change in the EPR line shape.[45,47] A strongly coupled biradical has also been observed in the ribonucleotide triphosphate reductase under rapid freeze-quench reaction conditions,[22] and has been identified as a Co^{II}-cysteine thiyl biradical.[13,24] This state is therefore an intermediate in transfer of radical character to the substrate, not a substrate-associated radical.[13,24] The preceding brief summary of EPR studies of vitamin B_{12} coenzyme enzymes shows that, with the exception of the non-native, 2-aminopropanol-derived unrearranged substrate radical in ethanolamine deaminase[21] and the native 4-glutamyl (unrearranged) substrate radical in glutamate mutase,[46] the identities of substrate-derived radical intermediate states are not known.

We have prepared and cryotrapped a weakly coupled biradical intermediate in ethanolamine deaminase from *Salmonella typhimurium* during turnover on the natural substrate, aminoethanol. Differences in the X-band EPR spectra among natural abundance, ^2H- and ^{13}C-labeled aminoethanol show that the $g = 2.0$ radical component of the spectrum is substrate-derived. Detailed comparison of the isotope-induced changes in the $g = 2.0$ line shape demonstrates that the radical represents the *rearranged* substrate radical, or "product radical". This is the first demonstration of a product radical intermediate in a vitamin B_{12} coenzyme-dependent enzyme. The observed steady-state accumulation of the product radical is combined with previous biochemical results to construct a qualitative free energy profile for the radical-associated reactions that provides insight into the mechanism of radical-mediated catalysis in ethanolamine deaminase.

Experimental Section

Enzyme Preparation. Enzyme was purified from the *Eschericia coli* overexpression strain incorporating the cloned *S. typhimurium* ethanolamine deaminase coding sequences[48] essentially as described,[49] with the exception that the enzyme was dialyzed against buffer containing 100 mM HEPES (pH 7.45), 10 mM KCl, 5 mM dithiothreitol, 10 mM urea, and 10% glycerol.[17] Enzyme activity was assayed as described[50] by using the sensitive 3-methylbenzothiazolinone hydrazone colorimetric method for measurement of acetaldehyde production.[51] The activity of the purified enzyme with aminoethanol as substrate was 20−30 μmol/min/mg.

(28) An illustrative systematic set of simulations of Co^{II}-radical EPR spectra has been performed for different electron−electron isotropic exchange and dipolar coupling strengths.[24]

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(32) The identification of the 2-aminopropanol-generated radical as the unrearranged substrate radical[21] was later questioned because electron spin−echo envelope modulation (ESEEM) spectra obtained from the radical generated from natural abundance ^{14}N-aminoethanol and ^{15}N-labeled aminoethanol were interpreted to be indistinguishable.[33] However, owing to the sensitive dependence of ESEEM amplitudes on the relative contributions of dipolar and isotropic hyperfine coupling contributions,[34] the absence of features from the substrate nitrogen in the ESEEM spectra does not constitute sufficient evidence that the substrate nitrogen is absent from the radical. The ESEEM results[33] are therefore consistent with the assignment[21] of the $g = 2$ EPR signal to the unrearranged, substrate radical.
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Sample Preparation. Adenosylcobalamin (Sigma Chemical Co.), unlabeled aminoethanol (Aldrich Chemical Co.), and isotopically-labeled aminoethanol (Cambridge Isotope Laboratories, Inc.) were purchased from commercial sources. The reaction was performed in buffer containing 100 mM HEPES (pH 7.45), 10 mM KCl, and 5 mM dithiothreitol. Identical results were obtained with air-saturated and anaerobic samples. Reaction was initiated by addition of adenosylcobalamin to premixed enzyme and substrate. All manipulations were carried out on ice in a cold room (273 K). The final concentration of enzyme was 20−30 mg/mL, which is equivalent to 40−60 μM for a holoenzyme molecular mass of 500000 g/mol, and 80−120 μM active sites.[49] Adenosylcobalamin was present at 80−120 μM (equimolar with active sites), and the concentration of aminoethanol substrate was 100 mM. Following thorough mixing, the samples were loaded into 4 mm o.d. EPR tubes and plunged into liquid nitrogen-chilled isopentane ($T \approx 130$ K) to freeze. The mixing, loading, and immersion in isopentane were accomplished in 12 s. The criteria of steady-state turnover at the time of cryotrapping are satisfied, as the following estimations show. The turnover number (k_{cat}) for aminoethanol in *S. typhimurium* ethanolamine deaminase is 22 s^{-1} (value of 55 s^{-1} at 295 K,[49] scaled to 273 K by the factor exp[−273/295]). For an initial aminoethanol concentration of 100 mM and 100 μM active sites, 26 mM substrate is consumed in 12 s, assuming that the initial rate is time-independent. In practice, a lag phase of several seconds precedes the rise to the steady state.[11a,23] This would result in less substrate consumption than estimated above.

We have also generated the biradical by mixing holoenzyme with aminoethanol, by using active site and stoichiometric adenosylcobalamin concentrations from 20 (aminoethanol, 20 mM) to 360 μM (aminoethanol, 380 mM), and by using a protocol involving mixing of reactants in the EPR tube that led to mixing and complete sample-tube immersion in isopentane at $T \approx 130$ K in 1 s. In all cases, the same biradical line shape was obtained.

Continuous-Wave EPR Spectroscopy. EPR spectra were obtained by using a Bruker ER200D EPR spectrometer equipped with a Bruker 4102ST/8216 TE cavity, HP 4256L frequency counter, Varian V3603 electromagnet, and Fieldial Mark I regulator/power supply, and Air Products cryostat and temperature controller modified for nitrogen gas flow sample cooling.

Results and Discussion

Generation of the Aminoethanol-Derived Radical. The radical intermediate present during steady-state turnover of substrate aminoethanol in *S. typhimurium* ethanolamine deaminase was prepared for EPR spectroscopy by cryotrapping enzyme immediately following mixing with active site-stoichiometric vitamin B_{12} coenzyme and excess substrate. Steady-state conditions were maintained through cryotrapping by preparing the samples at 273 K, which slowed enzyme turnover, thus minimizing substrate depletion and maximizing radical yield. A detailed description of the conditions and estimation of a substrate depletion of <25% prior to cryotrapping is given in the Experimental Section. Spin counts performed by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as standard showed that 0.20 organic radicals/active site are present when natural abundance aminoethanol is the substrate. The same radical was obtained at 295 K, but in much lower yield. If reaction was allowed to proceed for times leading to substrate depletion, the aminoethanol-derived radical signal was not observed. In addition, no radical was observed if ethanolamine deaminase, adenosylcob(III)alamin, or substrate were selectively removed from the reaction mixture. Further, radical formation was not observed if ethanolamine deaminase was incubated with hydroxocobalamin (5:1 excess over active sites), a powerful competitive inhibitor of adenosylcobalamin binding to ethanolamine deaminase,[11b,52] prior to the addition of adenosylcobalamin. These results demonstrate that the formation of the aminoethanol-derived radical is dependent upon catalytically competent ethanolamine deaminase operating under steady-state conditions.

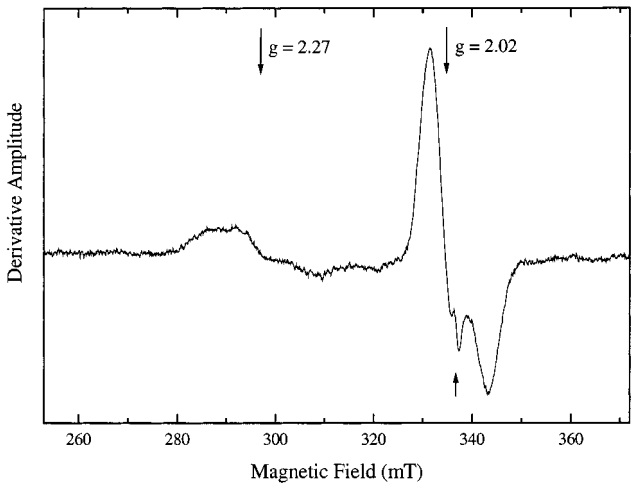

Figure 2. X-band EPR spectrum of the substrate-derived biradical in ethanolamine deaminase obtained for natural abundance aminoethanol substrate. The *g*-values at zero-crossing of the derivative line shapes in the Co^{II} $g_\perp$ and organic radical regions are shown. The arrow shows a narrow line width $g = 2$ radical signal that is present at low concentration in some samples. Conditions: Microwave power, 4 mW; microwave frequency, 9.456 GHz; magnetic field modulation, 1.0 mT; modulation frequency, 100 kHz; temperature, 120 K; scan rate, 0.6 mT/s; time constant, 200 ms; average of 8 scans minus baseline.

Biradical EPR Spectrum. Figure 2 shows the X-band EPR spectrum of the radical intermediate in ethanolamine deaminase obtained when natural abundance ethanolamine is the substrate. The low-field region of the spectrum shows a broad feature with a *g*-value at zero-crossing of 2.27, which is within the range of $g_\perp$ values of 2.2−2.3 reported for low-spin Co^{II} in cob(II)alamin in different environments.[29] The spectrum also displays a resonance characteristic of an organic radical in the $g = 2$ region with an absorption maximum at $g = 2.02$. The line width of the radical signal, which corresponds to the difference in resonant magnetic field values between the low-field peak and high-field trough of the absorption derivative line shape, is 10.8 mT.[53] This is broader than is typical of isolated organic radicals in the solid state.[54] The radical is also less readily microwave power-saturated ($P_{1/2} = 53$ mW, 155 K) than organic radicals that are not coupled to a paramagnetic metal ion (for example, $P_{1/2} = 0.2$ mW, 155 K, for 1,1-diphenyl-2-picrylhydrazyl in toluene). The broad line width and microwave power saturation behavior are similar to those of the well-characterized 2-aminopropanol-1-yl radical in *Clostridium* sp. ethanolamine deaminase,[21] which are reproduced in the *S. typhimurium* enzyme.[27] The line shapes of the 2-aminopropanol-1-yl and aminoethanol-generated radicals are compared in a separate report.[27] EPR spectral simulations of the 2-aminopropanol-1-yl substrate radical state in ethanolamine deaminase have shown that the

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(53) The 10.8 mT line width reported here is comparable with the line width of a previously studied aminoethanol-generated organic radical in ethanolamine deaminase.[35a] In a rapid freeze-quench study, quenching of mixtures of holoenzyme and aminoethanol under steady-state turnover conditions at 50−250 ms produced a radical with a line width of approximately 13 mT ($T = 120$ K).[36] As described in the Experimental Section, we obtain the 10.8 mT line width when aminoethanol is mixed with either apo- or holoenzyme, and over a range of protein and substrate concentrations, including those used in the previous study.[36] Rapid freeze-quench studies of the aminoethanol-generated radical trapped on the millisecond time scale are being pursued in an effort to explain the line shape differences (K. Warncke, C. Krebs, and B. H. Huynh, in progress).

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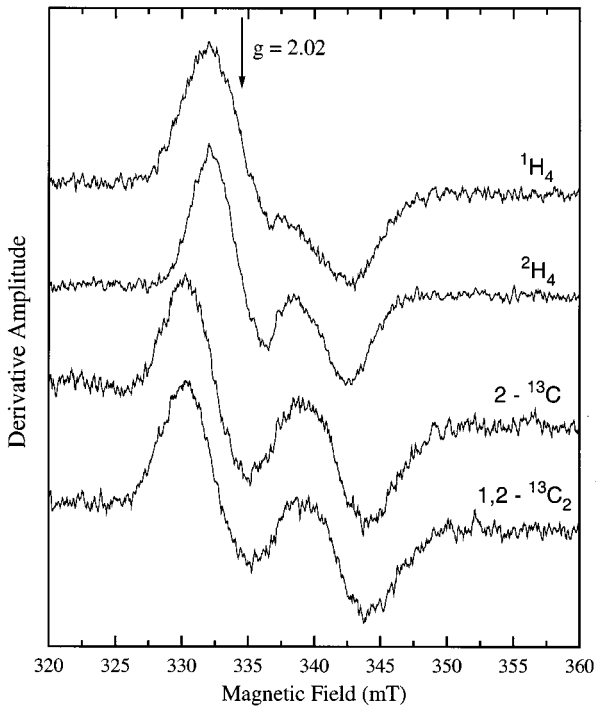

Figure 3. CW-EPR spectra of the substrate-derived radical in ethanolamine deaminase obtained for natural abundance and isotopically labeled aminoethanol substrates. Conditions: Microwave power, 1 mW; microwave frequency, 9.455 GHz; magnetic field modulation, 0.63 mT; modulation frequency, 100 kHz; temperature, 130 K; scan rate, 0.3 mT/s; time constant, 100 ms; average of 16 scans minus baseline.

broad Co^{II} resonance, which shows no resolved cobalt hyperfine features, and the relatively broad $g = 2$ radical signal arise from coupling of the substrate radical with Co^{II} in cob(II)alamin over a distance of ~10 Å through relatively weak electron spin−spin exchange and dipolar interactions.[26] The enhanced relaxation of the radicals is also caused by interaction with Co^{II}. These results show that the aminoethanol-derived radical intermediate and Co^{II} are spin-coupled. Thus, the $g = 2$ radical is located within the protein at the active site.[26,31]

The Radical Intermediate Is Derived from the Substrate. The aminoethanol-generated radical was prepared by using isotopically labeled substrates to identify a substrate versus protein or coenzyme origin of the intermediate. Figure 3 shows X-band EPR spectra of the radical intermediate generated from natural abundance and isotopically labeled aminoethanol. The zero-crossing of the spectrum of the radical derived from natural abundance aminoethanol is positioned at $g = 2.02$. The radical derived from 1,1,2,2-$^2H_4$-aminoethanol displays a narrower line width of 10.1 mT as well as a narrowing of the peak and trough features, in comparison with the line shape generated with natural abundance aminoethanol. The 0.7 mT line-narrowing and line shape changes relative to the natural abundance substrate are consistent with coupling of the unpaired spin with one or more of the four labeled hydrogen nuclei, since the strength of the hydrogen hyperfine coupling is decreased for ^2H versus ^1H by a factor of $\gamma_{1H}/\gamma_{2H} = 6.51$,[54] where γ is the nuclear gyromagnetic ratio. The line shape changes are muted from those expected for an isolated organic radical, because they are partially obscured by electron spin−spin exchange and dipolar broadening.[26,29,31] The radicals derived from 2-^{13}C- and 1,2-$^{13}C_2$-labeled aminoethanol exhibit a common line width of 13.2 mT. The line broadening of 2.4 mT relative to the diamagnetic ^{12}C-containing natural abundance substrate demonstrates an interaction of the unpaired electron spin with the nuclear spin $I = 1/2$ ^{13}C nucleus. These results establish that the unpaired spin density in the radical intermediate is localized on a substrate-derived fragment.

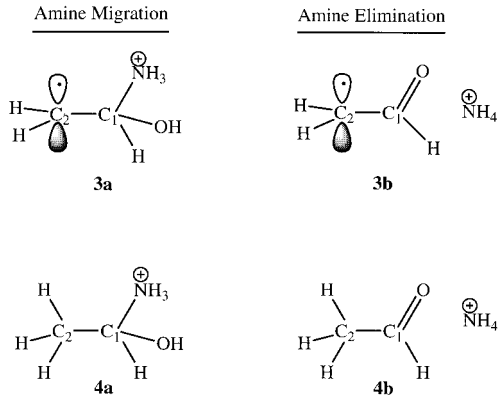

Figure 4. Intermediates proposed for the amine migration and amine elimination pathways of rearrangement in ethanolamine deaminase.[3,6,55] The full structures of the proposed intermediates correspond to the partial structures shown for species **3** and **4** in Figure 1.

The Radical Intermediate Is a Product Radical. The line widths of the radicals incorporating the 2-^{13}C and 1,2-$^{13}C_2$ substitutions are the same within the experimental signal-to-noise ratios. Therefore, the unpaired spin density is localized predominantly at a single position in the radical. The position corresponds to the amino-carbon atom, C_2, in the original substrate aminoethanol. The localization of the unpaired spin density at C_2 indicates that the aminoethanol-derived radical represents a rearranged, or product, radical state. As shown in Figure 1, this is because nitrogen elimination, or migration, must occur prior to localization of unpaired spin density at C_2. The direct identification of the product radical intermediate further authenticates the model of radical-mediated catalysis in ethanolamine deaminase that is schematized in Figure 1.

Proposed Rearrangment Mechanisms and the Structure of the Product Radical. Two pathways have been considered for the rearrangement reaction.[3,55] The full structures of the proposed intermediates in these two pathways are shown in Figure 4, and correspond to the partial structures of the product radical **3** and diamagnetic product **4** shown in Figure 1. In the amine migration pathway, ammonia departs from C_2 and readds at C_1 to form the 1-aminoethanol-2-yl radical **3a**, which reabstracts a hydrogen atom from 5′-deoxyadenosine to form 1-aminoethanol **4a**. Ammonia elimination occurs from **4a** to form product ethanal. The proposal of this pathway is based on the speculation that, following elimination of ammonia from C_2, the probability of ammonia readdition at the adjacent C_1 is enhanced by active site confinement,[3] and by the demonstration of this pathway in diol dehydrase.[56] The alternative amine elimination pathway proceeds through the ethanal-2-yl radical **3b** to products **4b**, and is supported by the prevalence of this route in model solution radical-mediated elimination reactions.[55,57]

The spectra presented in Figure 3 do not allow us to distinguish between intermediates **3a** and **3b** as the origin of the product radical. Both of these radicals incorporate ^2H from the $^2H_4$-labeled substrate in the same positions. A weak $^{13}C_1$ hyperfine coupling could be expected in **3a**, where sp^3-

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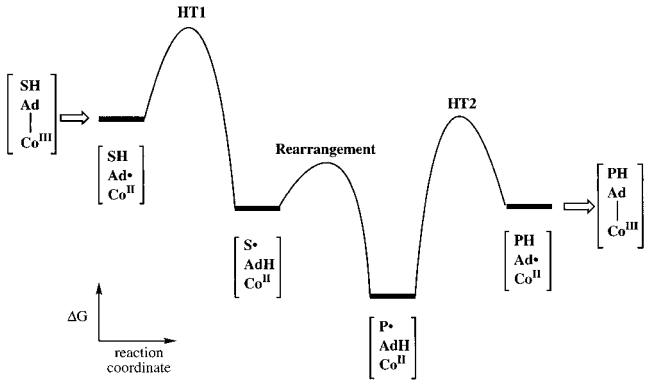

Figure 5. Free energy profile for biradical intermediate states in the reaction of aminoethanol with ethanolamine deaminase. The diamagnetic input and output states are also depicted for clarity, but without reference to free energy. The direction of the forward reaction is to the right. The relative positions of the free energy levels of each state and the relative barrier heights are derived as described in the text. The exact values for the free energy differences between states and for the barriers are unknown, with the exception of the HT2 barrier in the forward direction (15 kcal/mol).[6] The biradical intermediate states are linked by the HT1, rearrangment, and HT2 steps, which correspond to the same steps depicted in Figure 1. The chemical species within brackets corresponding to each state are symbolized as follows: Co^{III}/Co^{II}, cobalt in cobalamin in the indicated formal oxidation state; Ad-, 5′-deoxyadenosyl ligand; Ad•, 5′-deoxyadenosyl radical; AdH, 5′-deoxyadenosine; SH, aminoethanol; S•, substrate radical; PH, ethanal (+ammonia/ammonium); P•, product radical.

hybridization at C_1 would allow coupling only through dipolar interaction and σ-bond spin polarization[58] arising from unpaired p_π spin density at C_2. A weak $^{13}C_1$ hyperfine coupling could also be expected for **3b**, since solution EPR studies indicate a partial unpaired spin density of ≤ 0.05 at C_1.[57a] Therefore, the results are consistent with the product radical predicted in each pathway.

Energetics of Radical-Mediated Steps in Ethanolamine Deaminase. The direct detection of product radical **3** accumulation during steady-state turnover of aminoethanol by ethanolamine deaminase substantiates the interpretations of the results of previous isotope exchange studies obtained using this substrate. Figure 5 shows a qualitative reaction free energy profile for the biradical intermediate states in ethanolamine deaminase with aminoethanol as substrate, which is constructed from the EPR and isotope exchange results, as described below. From comparison of the deuterium kinetic isotope effects of 1,1-$^2H_2$-aminoethanol on the overall reaction rate[59] and on the rates of the first and second hydrogen transfer steps,[60] denoted by HT1 and HT2 in Figure 5, it was concluded that the HT2 step was rate limiting.[60] Therefore, HT2 is associated with the highest free energy barrier in the forward direction in Figure 5. An estimate of the height of this free energy barrier of 15 kcal/mol at 298 K was obtained from the temperature dependence of the maximum reaction velocity (V_m).[6] It was also proposed that HT1 and HT2 bracketted an irreversible step, based on the failure of tritium in substrate aminoethanol to equilibrate with the coenzyme during turnover.[60] The same step was proposed to be both rapid and irreversible based on the partitioning of tritium from 5′-^{3}H-labeled adenosylcob(III)alamin into only product ethanal, with no detection of tritium in substrate aminoethanol, upon reaction of holoenzyme with unlabeled aminoethanol.[61] From Figure 1, the rapid and irreversible step can be identified as the rearrangement step. These results[59−61] indicate that, once formed, the aminoethanol-generated substrate radical **2** is rapidly converted into the product radical **3**, which does not detectably back-equilibrate through **2** to **1**, despite the accumulation of the product radical state. As shown in Figure 5, this corresponds to a low free energy barrier for rearrangement relative to HT2, and significant lowering of the free energy of the product radical state relative to the substrate radical state. The accumulation of the product radical **3** verifies these features of the reaction free energy profile.

The relative free energy levels of the 5′-deoxyadenosyl radical-associated states in ethanolamine deaminase that are depicted in Figure 5 are less certain. Knowledge of the reversibility of HT1, which would allow an estimation of the relative free energies of the [Co^{II} Ad• SH] and [Co^{II} AdH S•] states in Figure 5, is obscured by the rapid and irreversible rearrangement step that follows the first hydrogen transfer.[61] The [Co^{II} Ad• SH] state does not detectably accumulate during turnover on aminoethanol, or on the substrate analogue, 2-aminopropanol,[21,27,62] for which the HT1 step is reversible[61] (the reaction with 2-aminopropanol is described in greater detail in the next section). This suggests that the [Co^{II} Ad• SH] state is higher in free energy than the [Co^{II} AdH S•] state. By analogy with the reversibility of the HT1 step for 2-aminopropanol,[61] we propose a relatively small free energy difference across the HT1 step. Therefore, in Figure 5, we place the [Co^{II} Ad• SH] state above, but near to, the [Co^{II} AdH S•] state in free energy, and give HT1 a barrier that is less than that for HT2 but greater than that for the rearrangement step. The HT1 and HT2 steps are similar in that each involves the Ad•/AdH hydrogen atom transfer couple and a carbon-based R•/RH couple. The HT2 step has been reported to be reversible for aminoethanol.[61,63] Therefore, we assume that the HT1 and HT2 barriers are mirror-symmetric about the free energy axis. This assumption depends critically on the similarity of the S•/SH and P•/PH couples, and may require some modification once the detailed structure of P• (and by inference, PH) is determined. The product state is likely to share some of the stabilization gained by the product radical, so that the [Co^{II} Ad• PH] state lies below the [Co^{II} Ad• SH] state in free energy.

Comparison with Substrate Radical Accumulation in the Reaction with 2-Aminopropanol. In contrast to the product radical accumulation observed with aminoethanol, reaction of ethanolamine deaminase with 2-aminopropanol leads to the accumulation of the 2-aminopropanol-1-yl *substrate* radical.[21,32] Our EPR results clarify this enigmatic result. The accumulation of the 2-aminopropanol-derived substrate radical was originally attributed to rate limitation at the rearrangement step.[61] This proposal was based on the ^{3}H-2-aminopropanol:^{3}H-propanal ratios of 2:1 and 0.3:1 when reaction of holoenzyme incorporating ^{3}H-coenzyme was begun with unlabeled substrate (2-aminopropanol; forward reaction conditions) and unlabeled products (propanal and ammonium ion; reverse reaction), respectively.[61] Subsequently, the deuterium kinetic isotope effect of 1-$^2H_2$-2-aminopropanol on the overall reaction rate showed that hydrogen transfer was at least partially rate-limiting.[64] Based principally on the similar substrate deuterium isotope effects

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(62) A unique line shape that is more akin to the strongly coupled Co^{II}-radical line shapes[28] is expected for the Co^{II}-deoxyadenosyl biradical, because the deoxyadenosyl is expected to be closer to the Co^{II} than the substrate or product radicals.
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on the overall reaction rate with 2-aminopropanol (5.9)[64] and aminoethanol (7.4),[61] it was proposed that the HT2 step was also rate limiting for reaction with 2-aminopropanol.[64] However, although the isotope effects are similar, the effect for 2-aminopropanol is less than that for aminoethanol, indicating the contribution of at least one other step not involving hydrogen transfer to the rate limitation. Our results show that, if HT2 is solely rate limiting, then the product radical should accumulate in the reaction with 2-aminopropanol. Therefore, the observed[21] accumulation of the substrate radical in the reaction with 2-aminopropanol must be caused by an increase in the barrier to rearrangement, a decrease in the free energy of the substrate radical relative to the product radical, or a combination of the two. The favored partitioning of tritium from ^{3}H-coenzyme into propanal versus 2-aminopropanol in the reverse versus the forward reaction suggests that the rearrangement is still thermodynamically favorable in the forward direction.[61] However, the rearrangement is less exothermic than for aminoethanol, because the reaction with 2-aminopropanol is fully reversible.[6,21,61,64] Therefore, the accumulation of the substrate radical in the reaction with 2-aminopropanol is caused by a combination of an increased barrier and a decrease in exothermicity for the rearrangement reaction.

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Product Radical Trap Mechanism in Ethanolamine Deaminase. We propose that a "product radical trap" strategy contributes to radical pair stabilization, and hence productive reaction, in ethanolamine deaminase. The product radical trap is characterized by a rapid, irreversible rearrangement step that effectively traps the radical pair state in the product radical state. On the reaction free energy profile shown in Figure 5, this corresponds to a low rearrangement barrier relative to the HT1 barrier in the reverse direction, and a significant drop in free energy across the rearrangment step. The importance of the product radical trap in ethanolamine deaminase performance is highlighted by the 2 orders of magnitude decrease in turnover number when the native reaction free energy profile shown in Figure 5 is perturbed by using 2-aminopropanol as the substrate.[6]

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