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Catalytic Roles of Substrate-Binding Residues in Coenzyme B_{12}-Dependent Ethanolamine Ammonia-Lyase

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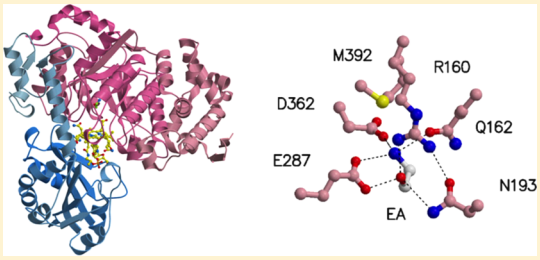
ABSTRACT: Ethanolamine ammonia-lyase (EAL) catalyzes the adenosylcobalamin-dependent conversion of ethanolamine to acetaldehyde and ammonia. 1-OH of the substrate is hydrogen-bonded with Gluα287, Argα160, and Asnα193 and 2-NH_2 with Gluα287, Glnα162, and Aspα362. The active site somewhat resembles that of diol dehydratase. All five residues were important for the high-affinity binding of the substrate and for catalysis. The -COO^- group at residue α287 was absolutely required for activity and coenzyme Co−C bond cleavage, and there was a spatially optimal position for it, suggesting that Gluα287 contributes to Co−C bond homolysis, stabilizes the transition state for the migration of NH_2 from C2 to C1 through partial deprotonation of spectator OH, and functions as a base in the elimination of ammonia. A positive charge and/or the hydrogen bond at position α160 and the hydrogen bonds at positions α162 and α193 with the substrate are important for catalysis and for preventing a radical intermediate from undergoing side reactions. Argα160 would stabilize the trigonal transition state in NH_2 migration by electrostatic catalysis and hydrogen bonding with spectator OH. Asnα193 would contribute to maintaining the appropriate position and direction of the guanidinium group of Argα160, as well. Hydrogen bond acceptors were necessary at position α162, but hydrogen bond donors were rather harmful. Glnα162 might stabilize the trigonal transition state by accepting a hydrogen bond from migrating NH_3^+. The activity was very sensitive to the position of -COO^- at α362. Aspα362 would assist Co−C bond homolysis indirectly and stabilize the trigonal transition state by accepting a hydrogen bond from migrating NH_3^+ and electrostatic interaction.



Adenosylcobalamin (AdoCbl) or coenzyme B_{12} is a naturally occurring organometallic compound that contains a unique cobalt−carbon (Co−C) bond. It acts as a cofactor for enzymatic radical reactions, including carbon skeleton rearrangements, heteroatom eliminations, and intramolecular amino group migrations.[1−7] AdoCbl-dependent rearrangement reactions involve the migration of a hydrogen atom from one carbon atom of the substrate to the adjacent carbon atom in exchange for a group X, which moves in the opposite direction[1−10] (Figure 1A). Ethanolamine ammonia-lyase (EAL, EC 4.3.1.7) or ethanolamine deaminase catalyzes such a type of reaction, i.e., the conversion of ethanolamine to acetaldehyde and ammonia (eq 1), where the migrating group X is an amino group on C2 of the substrate.[11]

$$H_3^+NCH_2CH_2OH \rightarrow CH_3CHO + NH_4^+ \quad (1)$$

Both enantiomers of 2-aminopropanol are very poor substrates and serve as potent suicide inactivators for EAL.[12,13] The enzyme was discovered in choline-fermenting *Clostridium* sp.[14] and then found in many bacteria that require exogenous vitamin B_{12} for growth on ethanolamine.[15] They include *Escherichia coli*,[16] *Klebsiella aerogenes*,[16] *Bacillus megaterium*,[17] *Salmonella typhimurium*,[18] etc.

The Co−C bond of AdoCbl is cleaved by the enzyme in a homolytic fashion forming an adenosyl radical and cob(II)-alamin (B_{12r}) (Figure 1A). The generated adenosyl radical abstracts a hydrogen atom from C1 of ethanolamine forming 5′-deoxyadenosine and a substrate-derived radical. The substrate radical then rearranges to a product radical with the migration of the NH_2 group from C2 to C1. The hydrogen back-abstraction by the product radical from 5′-deoxyadenosine, followed by elimination of ammonia, leads to the formation of a final product acetaldehyde. The mechanism of action of EAL has been extensively studied using purified clostridial and recombinant *S. typhimurium* enzymes.[3,7,19−21] Both enzymes are composed of two kinds of subunits, large (α) and small (β) subunits that are encoded by *eutB* and *eutC* genes, respectively. The subunit structure of EAL is $\alpha_6\beta_6$.[22,23]

Recently, we reported the cloning and expression of the genes encoding EAL of *E. coli* as well as some enzymological properties of the purified recombinant enzyme.[24] Although wild-type EAL undergoes aggregation (precipitation) and inactivation at high concentrations, such properties were improved by short N-terminal truncation and Cβ34S mutation of its β subunit. The catalytic properties and subunit structure of *E. coli* EAL are not affected by this truncation and mutation.

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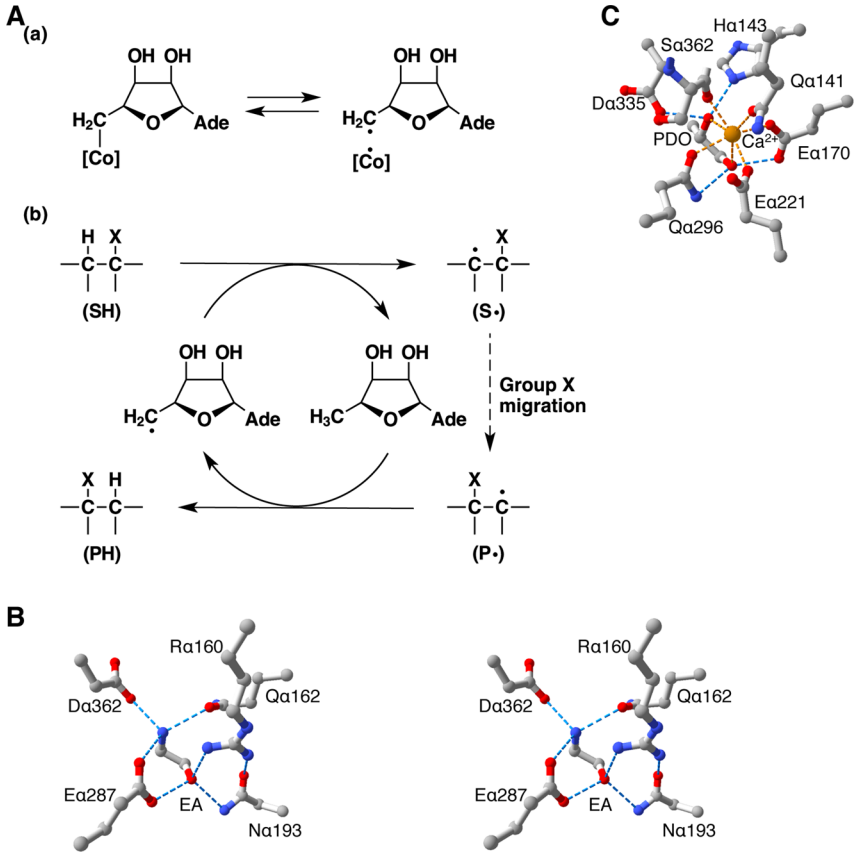

Figure 1. Minimal mechanism for AdoCbl-dependent enzymes (A) and active-site structures of EAL (B) and diol dehydratase (C). (A) Homolysis of the Co−C bond of enzyme-bound AdoCbl (a) and adenosyl radical-catalyzed rearrangements (b). Abbreviations: [Co], cobalamin; Ade, 9-adeninyl; SH, substrate; PH, product; X, generic migrating group (X = NH_3^+ on C2 of ethanolamine in the EAL reaction). Residue numbers in the α subunit. EA denotes ethanolamine in panel B. For the interaction with 2-aminopropanol, see refs 25 and 26. PDO denotes 1,2-propanediol in panel C.

We reported the X-ray structures of truncated EAL complexed with substrates (ethanolamine and 2-aminopropanol) and coenzyme analogues (cyanocobalamin and adeninylpentylcobalamin).[25,26] EAL exists as a $[(\alpha\beta)_2]_3$ trimer of the $(\alpha\beta)_2$ heterotetramer. The active site is located in the $(\beta/\alpha)_8$ or triosephosphate isomerase (TIM) barrel of the α subunit, and the OH and NH_2 groups of substrates are hydrogen-bonded to five amino acid residues, Argα160, Glnα162, Asnα193, Gluα287, and Aspα362 (Figure 1B). The β subunit covers the lower part of cobalamin that is bound in the interface of the α and β subunits in the base-on mode. On the basis of a comparative model for the structure of the α subunit of *S. typhimurium* EAL[27] and the properties of enzymes harboring Argα160 mutations,[28] Warncke and co-workers concluded that the positive charge at residue α160 is required for proper folding of the α subunit, assembly of a stable EAL oligomer, and catalysis in the assembled oligomer. Very recently, Bovell and Warncke reported the structural model for *S. typhimurium* EAL, based on the structure of the *E. coli* enzyme.[29]

In this work, we surveyed catalytic amino acid residues of *E. coli* EAL by alanine scanning. To understand the roles of these residues in EAL catalysis, mutant enzymes in which either substrate-binding residue is substituted with other amino acid residues were also prepared, and their catalytic properties were investigated by kinetic and spectroscopic analyses. During the preparation of this paper, Chen et al.[30] reported that Gluα287 is important in EAL for the generation and control of high-energy radical intermediates.

MATERIALS AND METHODS

Materials. AdoCbl was a gift from Eisai Co., Ltd. (Tokyo, Japan). Yeast alcohol dehydrogenase was obtained from Sigma-Aldrich (St. Louis, MO). Ni-NTA agarose was purchased from Qiagen GmbH (Hilden, Germany). All other chemicals were analytical grade reagents and used without further purification.

Construction of Expression Plasmids for Mutant EALs. Standard recombinant DNA techniques were performed as described by Sambrook et al.[31] Site-directed mutations were introduced into pET-21b(eutCΔCSHis$_6$-eutB), an expression plasmid for His$_6$-tagged, truncated EAL,[24] using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The primers used for introducing mutations are listed in Table 1. It was confirmed by sequencing of the DNA region encompassing the entire EAL genes that no unintended mutations had been incorporated during mutagenesis.

Expression and Purification of Mutant EALs. Recombinant *E. coli* BL21(DE3) carrying pET-21b(eutCΔCSHis$_6$-eutB) or its mutant plasmid was aerobically grown at 37 °C in LB medium containing ampicillin (50 μg/mL). When the culture reached an OD_{600} of approximately 0.9, isopropyl β-D-thiogalactopyranoside was added to a concentration of 0.1−1 mM for induction. After cultivation for a further 5.5 h, cells

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Table 1. Oligonucleotides Used for Site-Directed Mutagenesis[a]

mutation	sequence (sense primers)
Rα160A	gggcacctttagcgccgcgttgcagccaaatgac
Rα160K	ccgggcacctttagcgcc*aag*ttgcagccaaatgac
Qα162A	ctttagcgcccgtttggcgccaaatgacacccg
Qα162E	ctttagcgcccgtttgga*g*ccaaatgacacc
Qα162H	ctttagcgcccgtttg*cat*ccaaatgacaccc
Qα162K	ctttagcgcccgtttg*aag*ccaaatgacaccc
Nα193A	gcggtgatcggcgttgccccggtgactgac
Nα193D	gcggtgatcggcgttg*at*ccggtgactgac
Eα287A	ctgcctctacttc*gc*gaccggacaaggctc
Eα287D	ctgcctctacttc*gat*accggacaaggctc
Eα287H	ctgcctctacttc*cac*accggacaaggctctg
Eα287Q	ctgcctctacttc*caa*accggacaaggctc
Dα362A	catctctatgggctgtgcctgctgttataccaacc
Dα362E	catctctatgggctgtg*aat*gctgttataccaacc
Dα362N	catctctatgggctgt*aac*tgctgttataccaacc

[a]Italics indicate codons corresponding to substituted amino acid residues. The oligonucleotides having the complementary sequences in the opposite direction were used as the respective antisense primers.

were harvested, washed twice with 0.05 M potassium phosphate buffer (pH 8.0), and stored at −80 °C. Mutant apoenzymes were purified from cell-free extracts of overexpressing *E. coli* cells using a Ni-NTA agarose column, as described previously for His_6-tagged, truncated EAL.[24] The Superose 6 gel filtration step was omitted, because the enzymes that eluted from a Ni-NTA agarose column were almost homogeneous.

Enzyme and Protein Assays. EAL activity was routinely determined by the 3-methyl-2-benzothiazolinone hydrazone method using ethanolamine as the substrate.[24] One unit is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of acetaldehyde per minute at 37 °C under the standard assay conditions. For determining kinetic parameters, time courses of the EAL reaction were measured by the alcohol dehydrogenase−NADH coupled method using ethanolamine as the substrate.[24] k_{cat} was calculated from the maximal velocity that was determined by fitting the data to a straight line ($a + bx$). k_{inact} was determined by fitting the data to a decaying exponential $[y_0 + A \exp(-Bx)]$ using IGOR PRO (WaveMetrics, Inc., Lake Oswego, OR). Protein concentrations of purified enzymes were determined by measuring the absorbance at 280 nm. $\varepsilon_{M,280}$ calculated by the method of Gill and von Hippel[32] for His_6-tagged truncated EALs was 300960 M^{-1} cm^{-1}. $\varepsilon_{1\%,280}$ was calculated to be 6.33 on the basis of the predicted molecular weight.

PAGE. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was conducted as described by Laemmli.[33] Protein was stained with Coomassie Brilliant Blue R-250. SDS-7 (Sigma) molecular weight markers were used. Densitometric analysis of gels was conducted with a Printgraph AE-6911CX system (ATTO, Tokyo, Japan) and ImageJ version 1.47 (National Institutes of Health, Bethesda, MD).

Spectral Measurements. Wild-type or mutant apoEALs (1.0 mg, 2.1 nmol as $\alpha_6\beta_6$) in 0.9 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.3 or 0.01 M ethanolamine were incubated at 37 °C for 3 min in a cuvette. AdoCbl was added to a final concentration of 5 μM in a total volume of 1.0 mL. Optical spectra were recorded with a JASCO V-560 spectrophotometer 3, 10, and 30 min after the addition of AdoCbl. Enzymes were then denatured by adding 1.0 g of guanidine hydrochloride and 0.15 mL of 0.6 M citric acid. After incubation at 37 °C for 10 min, the mixture was neutralized to pH 8.0 by adding 0.2 mL of 1 M potassium phosphate buffer (pH 8.0) and 0.07 mL of 5 M KOH. After the spectral measurement, the mixture was photoirradiated in an ice−water bath for 5 min with a 250 W tungsten light bulb from a distance of 20 cm, and the spectrum was recorded again.

Electron Paramagnetic Resonance (EPR) Measurements. Substrate-free apoenzymes of wild-type and mutant EALs were obtained by dialysis against 33 volumes of 0.05 M potassium phosphate buffer (pH 8.0) containing 1% Brij35 for 38 h with two buffer changes. Apoenzyme (0.83 mg) was mixed at 0 °C with 50 nmol of AdoCbl in 0.6 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.9% Brij35 in a quartz EPR tube (outside diameter of 5 mm) stoppered with a rubber septum. After replacement of the air in the tube with argon by repeated evacuation and flushing, holoenzyme was formed by incubation at 25 °C for 3 min and then cooled to 4 °C. Ethanolamine or 2-aminopropanol (100 μmol) was added at 4 °C, and 1 min later, the reaction mixture was rapidly frozen in an isopentane bath (cooled to ca. −160 °C) and then in a liquid nitrogen bath. The sample was transferred to the EPR cavity that had been cooled with a cold nitrogen gas flow controlled by a Eurotherm B-VT 2000 temperature controller. EPR spectra were recorded at −130 °C on Bruker ESP-380E and ESP-300E spectrometers modified with a Gunn diode X-band microwave unit. The mixture was then incubated at 25 °C for 3 min and frozen for the second measurement. The mixture was incubated again for an additional 7 min and frozen for the third measurement. Finally, the mixture was incubated again for an additional 20 min and frozen for the fourth measurement. EPR conditions were as follows: microwave frequency, 9.471−9.486 GHz; modulation amplitude, 1 mT; modulation frequency, 100 kHz; microwave power, 10 mW.

RESULTS

Expression and Purification of Mutant EALs. In a previous paper, we reported that N-terminal truncation (residues 2−29) of the β subunit, C34S substitution of the β subunit, and addition of a C-terminal His_6 tag to the β subunit increased the solubility and/or stability of EAL without altering the catalytic properties.[24] A recently determined X-ray structure of EAL shows that the N-terminal region of the β subunit including residue 34 is away from the active site.[25] Therefore, we considered this engineered EAL as a wild-type enzyme (actually a truncated wild-type enzyme) and introduced mutations into its α subunit for functional analysis of substrate-binding residues. Mutant apoenzymes in which one of the substrate-binding residues, i.e., Argα160, Glnα162, Asnα193, Gluα287, or Aspα362 (Figure 1B), was substituted with Ala (Rα160A, Qα162A, Nα193A, Eα287A, or Dα362A, respectively) and chemically and/or structurally related or distinct amino acids (Rα160K, Qα162E, Qα162H, Qα162K, Nα193D, Eα287D, Eα287H, Eα287Q, Dα362E, and Dα362N) were expressed in *E. coli* and purified from cell-free extracts to homogeneity by Ni-NTA agarose column chromatography, as described previously for the truncated wild-type enzyme.[24] Upon SDS−PAGE, purified preparations of the mutant enzymes provided two bands corresponding to the α and truncated β subunits in a molar ratio of ∼1:1, as the truncated wild-type enzyme did (Figure 2). The results indicate that these mutations do not significantly alter the folding or subunit composition of EAL.

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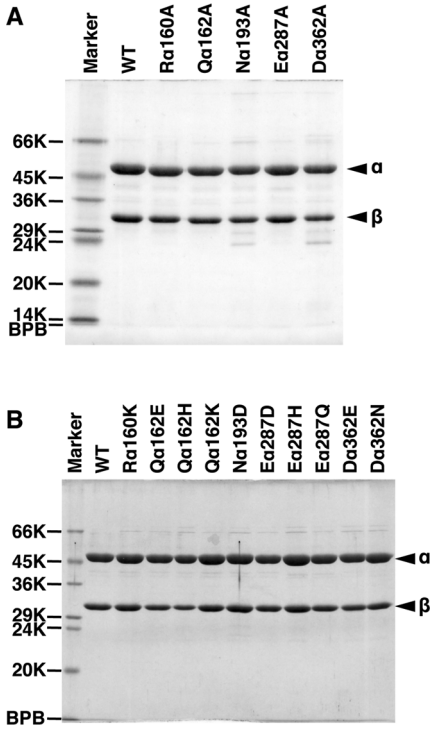

Figure 2. Analysis of the purified preparations of truncated wild-type and mutant EALs by SDS–PAGE: (A) Ala mutants and (B) other mutants. Samples were electrophoresed on 11% polyacrylamide gels. BPB denotes bromophenol blue and wt the wild-type enzyme. Positions of the α and β subunits are indicated at the right.

Catalytic Activity and Kinetic Properties of Mutant EALs. Time courses of ethanolamine deamination catalyzed by mutant EALs were measured by the alcohol dehydrogenase–NADH coupled method (data not shown), and the k_{cat} and k_{inact} values for mutant enzymes were determined from the maximal velocity and curve fitting, respectively. As shown in run 1 of Table 2, the substitution of either of the substrate-binding residues with Ala resulted in the loss of catalytic activity (k_{cat} less than 3% of that of the wild type), indicating that all five substrate-binding residues are catalytically important. EAL tends to undergo gradual inactivation during catalysis (mechanism-based inactivation) with ethanolamine as the substrate, following the first-order reaction kinetics (k_{inact} = 0.12 min^{-1} at 37 °C). Among the five Ala-substituted mutants, Qα162A, Nα193A, and Rα160A underwent inactivation 58–117 times more quickly than the wild-type enzyme. The k_{cat}/k_{inact} values indicate the average numbers of catalytic turnovers before inactivation. The k_{cat}/k_{inact} values for the five Ala-substituted mutants were in the range of 7.1–114, and these values are much smaller than that for the wild type (4.0×10^5), suggesting the strong tendency of the mutants to be inactivated during catalysis.

As indicated in run 2 of Table 2, mutant enzymes in which either of the substrate-binding residues is mutated to a related amino acid residue retain partial activity. Rα160K was 16% as active as the wild-type enzyme, suggesting the importance of a positive charge and/or a hydrogen bonding interaction with the 1-OH group of ethanolamine at this residue. This is consistent with the result obtained by Sun et al. with *S. typhimurium* EAL.[28] Nα193D was 7.3% as active as the wild type, indicating the importance of the hydrogen bonding interaction at this residue with the 1-OH group of substrate. Qα162E and Qα162H exhibited 22 and 1.2% of the wild-type activity, respectively, whereas Qα162K was totally inactive. This may indicate that the hydrogen bonding interaction at this residue with the 2-NH_2 group of the substrate is important for catalysis. k_{cat}/k_{inact} values for these partially active mutants were in the range from 740 to 9.7×10^3, which are smaller than 2.0×10^5 for the wild type, but much larger than those for the Ala-substituted mutants. Therefore, it was concluded that the positive charge and the hydrogen bond at residue α160 and the hydrogen bonds at residues α162 and α193 with the substrate are important for preventing a reactive radical intermediate

Table 2. Kinetic Parameters of Wild-Type and Mutant EALs[a]

run	enzyme	k_{cat} (s^{-1})	k_{inact} (min^{-1})	k_{cat}/k_{inact}	$K_{m(AdoCbl)}$ (μM)	$K_{m(ethanolamine)}$ (μM)
1	wild type	770	0.12	4.0×10^5		
	Rα160A	1.0	6.9	8.8		
	Qα162A	20	14	82		
	Nα193A	1.3	11	7.1		
	E$\alpha\alpha$287A	0.036	0.046	47		
	Dα362A	0.085	0.044	114		
2	wild type	490 ± 50	0.14 ± 0.00	2.0×10^5	0.77 ± 0.40	3.3 ± 2.3
	Rα160K	79 ± 2	1.2 ± 0.0	3.9×10^3	0.17 ± 0.014	210 ± 20
	Qα162E	110 ± 0	0.66 ± 0.00	9.7×10^3	0.79 ± 0.41	730 ± 140
	Qα162H	6.0 ± 0.2	0.49 ± 0.02	740	0.49 ± 0.13	220
	Qα162K	0.051 ± 0.006	0.16 ± 0.02	18		
	Nα193D	36 ± 1	0.40 ± 0.01	5.4×10^3	0.16 ± 0.015	210 ± 50
	Eα287D	2.1 ± 0.1	0.89 ± 0.01	143		
	Eα287H	0.066 ± 0.02	0.15 ± 0.00	26		
	Eα287Q	0.086 ± 0.001	0.14 ± 0.00	37		
	Dα362E	0.67 ± 0.03	0.14 ± 0.00	300		
	Dα362N	0.038 ± 0.009	0.14 ± 0.01	16		

[a]Kinetic parameters were determined by the alcohol dehydrogenase–NADH coupled method.[24] An assay mixture contained 0.0075 unit of apoenzyme, 15 μM AdoCbl, 15 mM ethanolamine, 0.2 mM NADH, 0.05 mg/mL yeast alcohol dehydrogenase (Sigma), and 0.04 M potassium phosphate buffer (pH 8.0), in a total volume of 1.0 mL. The reaction was started by the addition of AdoCbl. The oxidation of NADH to NAD^+ with acetaldehyde at 37 °C was monitored by measuring ΔA_{340}. k_{cat} and k_{inact} were obtained as described in the text.

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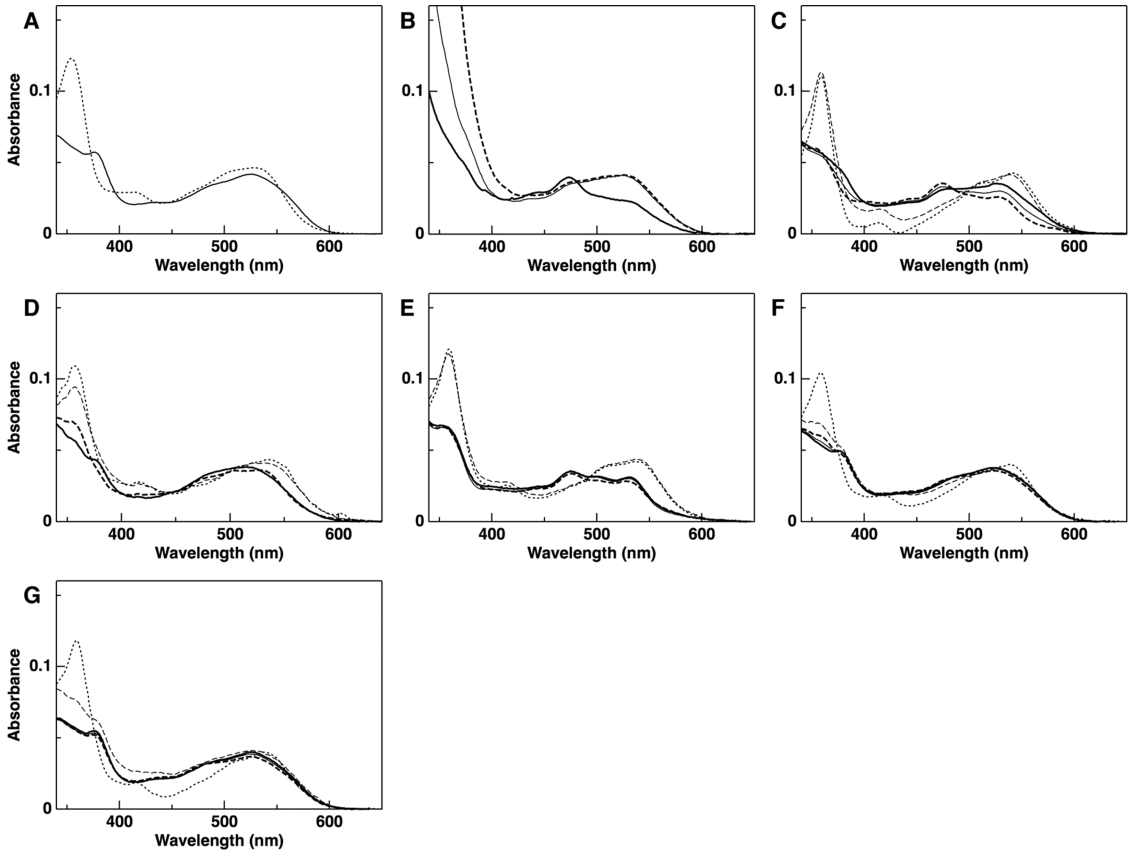

Figure 3. Spectral changes of AdoCbl upon incubation with wild-type EAL or Gluα287 mutant EALs in the presence of ethanolamine. (A) The spectra for free AdoCbl (5 μM) (solid line) and AdoCbl after photolysis (dotted line) in 0.05 M potassium phosphate buffer (pH 8.0) containing 0.01 M ethanolamine. (B–G) The substrate concentration was 0.01 M unless otherwise indicated. Spectra were recorded 3 min (thick solid lines), 10 min (thin solid lines), and 30 min (thick dashed lines) after the addition of AdoCbl, as described in the text. The spectra after denaturation (thin dashed lines) and photoirradiation (dotted lines) after incubation for 30 min were also recorded. Spectra were corrected for dilution: (B) wild type (0.3 M substrate), (C) wild type, (D) Eα287A, (E) Eα287D, (F) Eα287H, and (G) Eα287Q.

from undergoing side reactions that lead to mechanism-based inactivation of EAL. In contrast, the substitution of Gluα287 or Aspα362 with a neutral or basic residue abolished the EAL activity, while Eα287D and Dα362E retained a trace of activity (0.1–0.4% of the wild-type activity). It is thus evident that that the -COO^- groups are required for activity at the side chains of residues α287 and α362 and also that their spatial positions are critically important.

K_m values for AdoCbl and ethanolamine were determined with partially active mutants, i.e., Rα160K, Qα162E, Qα162H, and Nα193D (Table 2). K_m values of these mutants for AdoCbl were in the range of 0.17–0.79 μM, which are similar to that of the wild-type enzyme (0.77 μM) or even smaller. These results indicate that the mutations of Argα160, Glnα162, and Asnα193 did not significantly affect the binding affinity for the coenzyme. In contrast, K_m values of these mutants for ethanolamine were in the range of 0.21–0.73 mM, which are 64–220 times larger than that of the wild type (3.3 μM). It was therefore suggested that hydrogen bonding interactions with the side chains of Argα160, Glnα162, and Asnα193 participate in the high-affinity binding of the substrate.

Spectral Changes of AdoCbl upon Incubation with Mutant EALs. As shown in Figure 3B, AdoCbl undergoes spectral changes upon incubation with wild-type apoEAL in the presence of 0.3 mM ethanolamine. The absorbance at 526 nm decreased, and a new peak at 475 nm appeared after incubation for 3 min. The obtained spectrum reflects a high steady-state concentration of cob(II)alamin. Upon prolonged incubation, the substrate is exhausted within 10 min, and alkylcobalamin/hydroxocobalamin-like $\alpha\beta$ bands and an intense absorption band appeared in the UV region. The latter band might be due to the reaction of ethanolamine with acetaldehyde, because in nonenzymatic model experiments, such a band was observed by incubation of ethanolamine with acetaldehyde, but not by incubation of ammonia with acetaldehyde or in the presence of either ethanolamine or acetaldehyde alone (data not shown). In contrast, the steady-state concentration of cob(II)alamin was much lower in the presence of 10 μM substrate because of the exhaustion of the substrate within 3 min (Figure 3C); however, the magnitude of the peak at 475 nm gradually increased and the absorbance around 526 nm decreased by prolonged incubation, suggesting an increasing concentration of cob(II)-alamin. Because the enzyme is completely inactivated after incubation for 30 min, the finally obtained, cob(II)alamin-like spectrum would be that of inactivated holoEAL. Upon denaturation of the enzyme at this time, the spectrum of aquacobalamin/hydroxocobalamin was obtained, and it did not undergo further spectral change by photoirradiation. This indicates that the Co–C bond had already been cleaved completely in the irreversible inactivation during catalysis at this time.

When Eα287D was used instead of wild-type EAL, a similar spectrum was observed, although the spectral change was faster than that with the wild type (Figure 3E). This is consistent with

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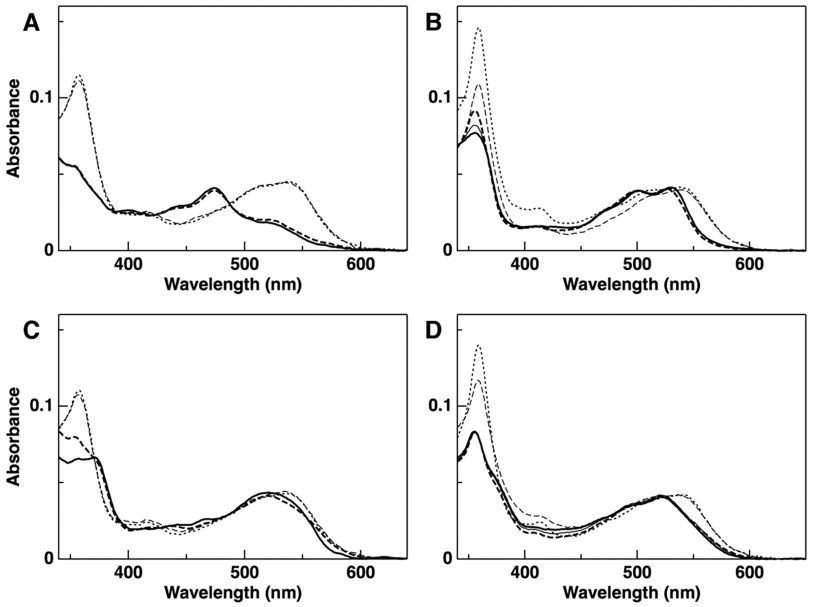

Figure 4. Spectral changes of AdoCbl upon incubation with Argα160 or Asnα193 mutant EALs in the presence of ethanolamine. Experimental conditions are the same as those described in the legend of Figure 3: (A) Rα160A, (B) Rα160K, (C) Nα193A, and (D) Nα193D.

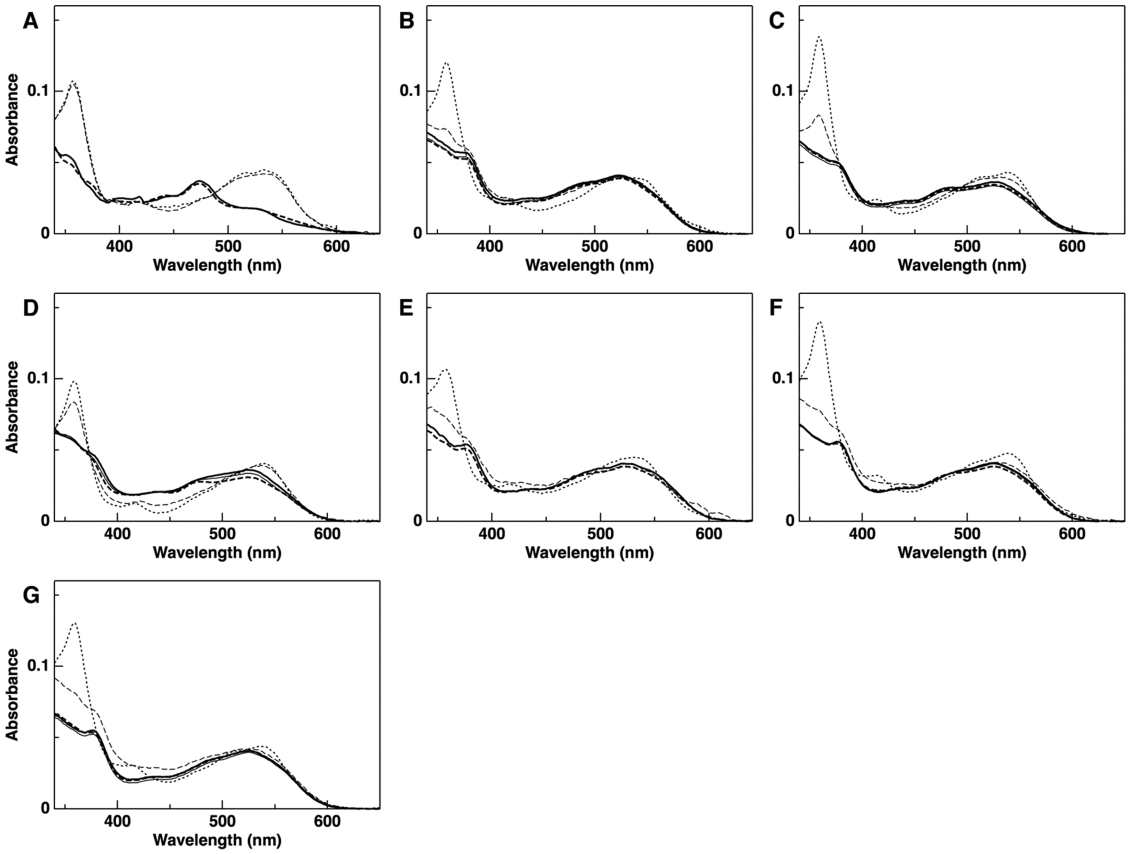

Figure 5. Spectral changes of AdoCbl upon incubation with Glnα162 or Aspα362 mutant EALs in the presence of ethanolamine. Experimental conditions were the same as those described in the legend of Figure 3: (A) Qα162A, (B) Qα162E, (C) Qα162H, (D) Qα162K, (E) Dα362A, (F) Dα362E, and (G) Dα362N.

the fact that this mutant shows a trace of EAL activity but becomes inactivated 6 times faster than the wild type. In contrast, major spectral changes were not seen with the Eα287A, Eα287H, and Eα287Q mutants (Figure 3D,F,G). These results indicate that the -COO^- group is required at residue α287 for catalytic activity and for the cleavage of the Co−C bond of AdoCbl, and that there is a spatially optimal position for it. Such a conclusion would be reasonable, because Gluα287 forms hydrogen bonds with both the 1-OH and 2-NH_2 groups of the substrate. Gluα287 is not fixed at the active

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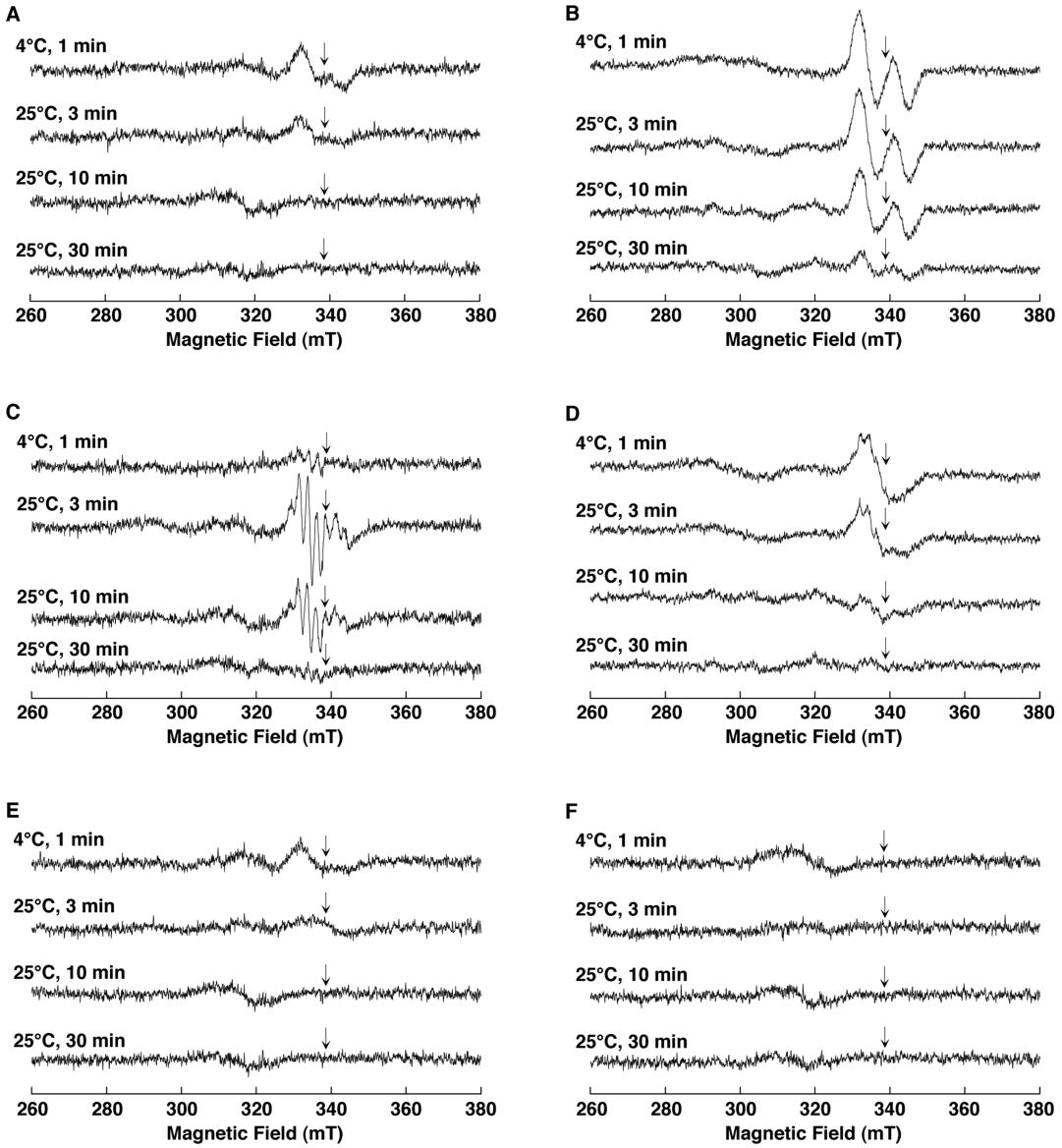

Figure 6. EPR spectra of the holoenzymes of wild-type EAL and its partially active mutants reacting with substrates. Experimental conditions are described in the text. The substrate used was 2-aminopropanol unless otherwise indicated. The positions of $g = 2.0$ are denoted with arrows: (A) wild type (ethanolamine as the substrate), (B) wild type, (C) Eα287D, (D) Rα160K, (E) Nα193D, and (F) Qα162E.

site in the substrate-free form, but fixed by the interaction with the substrate, which results in stabilization of the posthomolysis state through direct contact with the ribose OH group on C2′ and thus in substrate-induced additional labilization of the Co−C bond.[25]

Figure 4 shows the spectral changes of AdoCbl upon incubation with apoEALs mutated at Argα160 and Asnα193, both of which are hydrogen-bonded to the 1-OH group of the substrate. Upon incubation with Rα160A, AdoCbl was converted to cob(II)alamin within 3 min of incubation (Figure 4A). This is consistent with the fact that a trace of enzymatic activity of this mutant is lost 58 times more rapidly than the activity of the wild type. In contrast, the spectrum of AdoCbl was gradually changed to the hydroxocobalamin-like one upon incubation with Rα160K (Figure 4B), Nα193A (Figure 4C), or Nα193D (Figure 4D). These mutants retained 16, 0.2, and 7.3%, respectively, of the activity of the wild-type enzyme and were inactivated 3−92 times faster than the wild type. It is intriguing that the spectrum of the accumulated hydroxocobalamin-like species with Rα160K looks very similar to that of hydroxocobalamin bound to diol dehydratase together with 5′-deoxyadenosine.[34] These results suggest that the hydrogen bonds between the 1-OH group of the substrate and Argα160 and Asnα193 are not essential for Co−C bond cleavage, but the presence of a positive charge at residue α160 and the hydrogen bonding interaction of the substrate 1-OH group with residues α160 and α193 play important roles in preventing the reactive radical intermediates from undesirable side reactions, that is, negative catalysis.[35]

ApoEALs mutated at Glnα162 and Aspα362, both of which are hydrogen-bonded to the 2-NH_2 group of the substrate, were also incubated with AdoCbl, and the spectral changes were investigated. Upon incubation with Qα162A, the spectrum of AdoCbl changed to that of cob(II)alamin within 3 min of incubation (Figure 5A). This is also consistent with the fact that a trace of enzymatic activity of this mutant is lost 117 times more rapidly than the activity of the wild type. When Glnα162 was mutated to Glu, 22% of the activity was retained, and the

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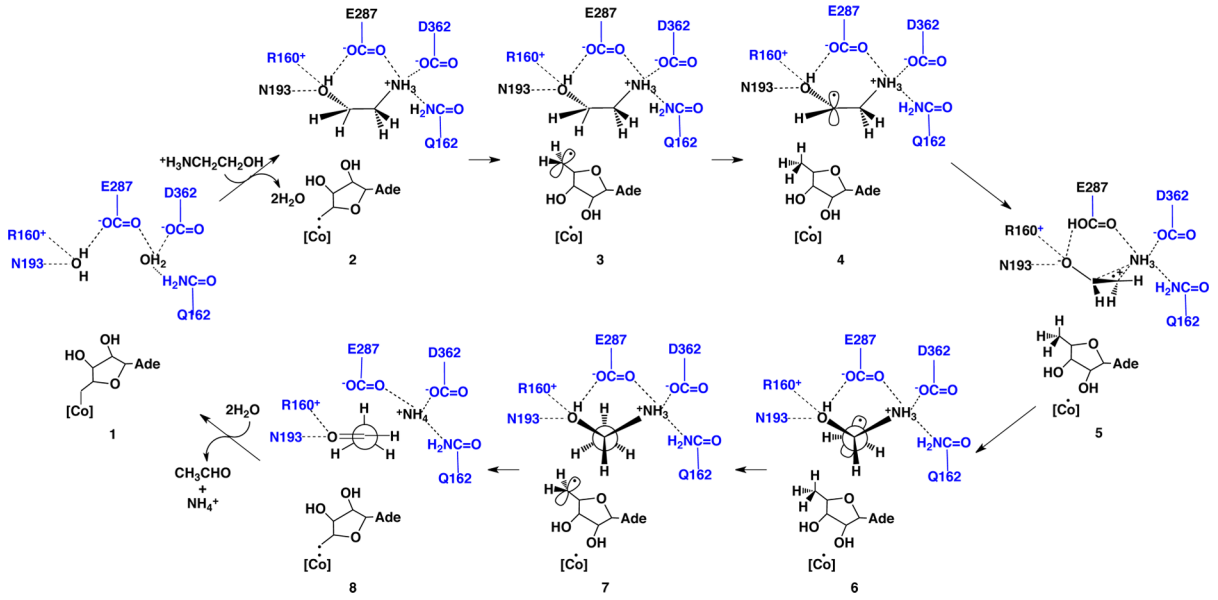

Figure 7. Refined overall mechanism for EAL and roles of substrate-binding residues in the reaction. [Co] denotes cobalamin and Ade the 9-adeninyl group. Amino acid residues of EAL are colored blue. Residue numbers are in the α subunit.

spectrum of AdoCbl did not change significantly during incubation (Figure 5B). This is consistent with the slow rate of inactivation of this mutant during catalysis. In contrast, when Glnα162 was mutated to His or Lys, changes in the spectra of AdoCbl were not significant (Figure 5C,D). These results indicate that the hydrogen bonding interaction of the 2-NH_2 group of the substrate with residue α162 is not essential for Co−C bond cleavage, but important for catalysis. On the other hand, AdoCbl did not undergo spectral changes upon incubation with EALs in which Aspα362 was mutated to Ala (Figure 5E), Glu (Figure 5F), or Asn (Figure 5G). It was concluded that the presence of the -COO^- group at residue α362 is essential for catalysis. Like Gluα287, Aspα362 might be essential for substrate binding and thus for substrate-induced additional labilization of the Co−C bond of the coenzyme.

EPR Spectra of Reacting Holoenzymes of Mutant EALs. To gain information about reaction intermediates, EPR spectra of reacting holoenzymes were measured (Figure 6). Upon incubation of the wild-type holoenzyme with ethanolamine and 2-aminopropanol at 4 °C, EPR spectra shown in panels A and B, respectively, of Figure 6 were obtained. Similar spectra were observed previously with full-length wild-type holoEALs of *E. coli*,[24] *Clostridium* sp.,[36] and *S. typhimurium*.[37−39] The high-field signals with a g value of ∼2 were identified as the ethanolamine-1-yl and 2-aminopropanol-1-yl radicals (substrate-derived radicals), and the low-field broad signals were assigned to low-spin Co(II) of cob(II)alamin.[36,38,39] Such spectra indicate the weak interaction in the Co(II)−organic radical pair,[40−42] although the doublet signal of the substrate-derived radical was less intense and its splitting less clear with ethanolamine (Figure 4A). The intensity of signals of substrate-derived radicals decreased with time of incubation at 25 °C with both substrates, which reflects the exhaustion of the substrate.

The EPR spectra of mutant holoEALs reacting with 2-aminopropanol were also measured using mutants that retain partial activity or a trace (in Figure 6). Eα287D (0.4% as active as the wild type) provided a complex $g \sim 2$ signal after incubation for 3 min at 25 °C (Figure 6C). Its signal intensity was very low after incubation for 1 min at 4 °C, decreased gradually with time of incubation, and almost disappeared after incubation for 30 min at 25 °C. At present, the radical species that gives this signal has not been identified. Upon incubation of Rα160K (16% as active as the wild type) with 2-aminopropanol, low-field broad signals and high-field intense signals were observed (Figure 6D). The latter signal would be due to the formation of organic radical(s), but its shape was different from that of the typical 2-aminopropanol-1-yl radical. The signal of the substrate radical might be contaminated by those of other organic radical(s). Nα193D (7.3% as active as the wild type) also showed some $g \sim 2$ signal after incubation for 1 min at 4 °C with 2-aminopropanol (Figure 6E). This signal disappeared with time of incubation, but again, the shape of the signal is not typical of the 2-aminopropanol-1-yl radical. These results might indicate that the geometries or binding conformations of substrate-derived radicals at the active sites of these mutants are somewhat different from that of wild-type EAL. Although Qα162E showed the highest EAL activity (22% as active as the wild type), it did not provide the $g \sim 2$ signal at all (Figure 6F). This might be because the steady-state concentration of the substrate radical is very low, which is consistent with a negligible cob(II)alamin concentration suggested by optical spectra with this mutant (Figure 5B).

DISCUSSION

The X-ray structure of EAL revealed that the substrate ethanolamine is bound to the enzyme by six hydrogen bonds with side chains of five active-site residues in the TIM barrel, that is, the 1-OH group with Gluα287, Argα160, and Asnα193 and the 2-NH_2 group with Gluα287, Glnα162, and Aspα362. These amino acid residues are highly conserved among EALs of many bacteria. The substrate-binding site of EAL (Figure 1B) somewhat resembles that of diol dehydratase[43,44] (Figure 1C). Because both EAL and diol dehydratase catalyze the same type of AdoCbl-dependent isomerization (eliminating) reactions, it might not be surprising that their active sites are similar. We surveyed catalytic amino acid residues of *E. coli* EAL by alanine scanning and found that all five substrate-binding residues are

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essential for catalytic activity. The roles of these residues in EAL catalysis were investigated by site-directed mutagenesis of the substrate-binding residues.

Gluα287 forms hydrogen bonds both with the 1-OH and 2-NH_2 groups of substrates. This residue is not fixed at the active site in the substrate-free form, but fixed by the interaction with substrates.[25] From the X-ray structure, it was postulated that the interaction with the substrate results in stabilization of the posthomolysis state through the direct contact with the ribose 2′-OH group of the coenzyme and thus in substrate-induced additional labilization of the coenzyme Co−C bond.[25] The findings that the -COO^- group at residue α287 is absolutely required for catalytic activity and for the cleavage of the Co−C bond and that there is a spatially optimal position for it are consistent with this idea. As depicted in Figure 7, Gluα287 would (i) participate in the binding of the substrate and all the intermediates throughout the reaction, (ii) contribute to the homolysis of the Co−C bond, (iii) stabilize the transition state for the migration of the NH_2 group from C2 to C1 through partial deprotonation of the spectator OH group ("pull effect"), and (iv) function as a base in the deamination of an aminocarbinol intermediate. If the position of the -COO^- group is not adequate as in Eα287D, the activity is very much lowered and the enzyme undergoes more inactivation during catalysis by side reactions of reactive radical intermediates. Scrutton and co-workers also reported that Gluα287 apparently provides both electrostatic and dynamic contributions to the Co−C bond homolysis and subsequent radical control in EAL.[30] A synergistic action of partial protonation of the NH_2 group and partial deprotonation of the OH group by His and Asp/Glu was proposed by calculations to lower the barrier of the NH_2 group migration catalyzed by EAL.[45,46] In diol dehydratase, evidence of the importance of partial deprotonation of the spectator OH group by Gluα170 for the transition-state stabilization in the OH group migration was obtained by experimental[47] and computational studies.[48,49] However, there are arguments about a substrate switch in EAL. Scrutton and co-workers discussed the dynamic substrate trigger for Co−C bond homolysis[50] and protein motions coupled to the reaction chemistry in EAL.[51,52] Warncke and co-workers reported that AdoCbl is not significantly distorted in the holoEAL−substrate complex,[53,54] and that substrate binding does not switch the protein to a new structural state.[54]

Argα160 and Asnα193 also form hydrogen bonds with the 1-OH group of substrates. Argα160 occupies a position similar to that of the metal ion in diol and glycerol dehydratases. This metal ion of diol dehydratase was first assigned to K^{+}[43] but recently reassigned to Ca^{2+}.[55,56] It is coordinated by the two OH groups of substrates and participates in lowering the activation barrier for the migration of the OH group from C2 to C1 through its stronger Lewis acidity in diol dehydratase.[57] In EAL, Rα160A was inactive, whereas Rα160K was 16% as active as the wild-type enzyme, suggesting the importance of a positive charge at this residue. This is consistent with the result obtained by Sun et al. with *S. typhimurium* EAL.[28] The positive charge in this position might be necessary to keep the net charge of zero for the transition state to lower its energy, a possibility that should be tested by theoretical calculations. As shown in Figure 7, Argα160 would (i) participate in the binding of the substrate and all the intermediates throughout the reaction and (ii) stabilize the trigonal transition state (**5**) in the NH_2 group migration by electrostatic catalysis as well as hydrogen bonding with the spectator OH group. Asnα193 occupies the position of Glnα296 in diol dehydratase. Although the Qα296A mutant of diol dehydratase is 52% as active as the wild type,[47] Nα193A EAL was inactive, while Nα193D retained 7.3% of the activity of the wild type. This is probably because Asnα193 forms hydrogen bonds not only with the 1-OH group of substrates but also with the guanidinium group of Argα160. Thus, this residue is supposed (i) to participate directly in the binding of the substrate and all the intermediates and (ii) to contribute to maintaining the appropriate position and direction of the guanidinium group of Argα160, which is also important for the binding of the substrate in the proper orientation.

Glnα162 and Aspα362 form hydrogen bonds with the 2-NH_2 group of substrates. This pair corresponds to the pair of Hisα143 and Aspα335 in diol dehydratase but shares the spatially reversed positions. Although Qα162A was only 2.6% as active, Qα162E was 22% as active as the wild type. In contrast, the mutants in which Glnα162 was substituted with His or Lys were almost inactive (1.2 or 0.0%, respectively). This might show that hydrogen bond acceptors, such as NH_2CO- and -COO^-, are necessary in this residue, but hydrogen bond donors, such as -NH_3^+ and imidazolium, are rather harmful. Therefore, as shown in Figure 7, Glnα162 would (i) participate in the binding of the substrate and all the intermediates and (ii) stabilize the trigonal transition state by accepting a hydrogen bond from the migrating NH_3^+ group. In the case of diol dehydratase, the importance of the hydrogen bond with Hisα143 was shown experimentally[58] and by theoretical calculations.[48,49] The Hα143A mutant of diol dehydratase is almost inactive (1.4% of the activity of the wild type), whereas Hα143Q is 34% as active as the wild type. This is in clear contrast with EAL, which is very interesting and might reflect the electronic natures of the transition state and the migrating group. It should also be noted that, if the hydrogen bonding interaction with substrate is not adequate as in Qα162H, the activity is very much lowered and the enzyme undergoes more inactivation during catalysis by side reactions of reactive radical intermediates. Aspα362 is also a very important catalytic residue. Dα362A, Dα362E, and Dα362N, the three Aspα362 mutants, were catalytically inactive. No cleavage of the coenzyme Co−C bond was observed with these mutants by optical and EPR spectroscopies. The inactivity of Dα362E indicates that the enzyme activity is very sensitive to the position of -COO^- at this residue. Aspα335 in diol dehydratase, corresponding to Aspα362 in EAL, is also an essential residue, and its mutant Dα335N is totally inactive,[47] suggesting that these Asp residues play similar roles in diol dehydratase and EAL. As illustrated in Figure 7, Aspα362 would (i) participate in the binding of the substrate and all the intermediates, (ii) assist Co−C bond homolysis indirectly, and (iii) stabilize the trigonal transition state by accepting a hydrogen bond and electrostatic interaction.

As discussed above, the active sites of EAL and diol dehydratase are distinct but share some characteristics in common. If we consider the similarity of the reactions they catalyze, it might not be surprising that their substrate-binding residues share the same roles in catalysis. Of course, many enigmas remain. The metal ion in diol dehydratase is not present in EAL and might be substituted with Argα160; Hisα143 in the former is substituted with Glnα162 in the latter, and so on. It seems interesting whether such differences are accidental or evolutionarily inevitable for these enzymes to

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address the different natures of the transition states and the migrating groups.

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Notes

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ABBREVIATIONS

AdoCbl, adenosylcobalamin or coenzyme B_{12}; EAL, ethanolamine ammonia-lyase; EPR, electron paramagnetic resonance; His_6, hexahistidine; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

REFERENCES

(1) Banerjee, R., Ed. (1999) *Chemistry and Biochemistry of B_{12}*, John Wiley & Sons, New York.

(2) Dolphin, D., Ed. (1982) *B_{12}*, Vol. 2, John Wiley & Sons, New York.

(3) Toraya, T. (2014) Cobalamin-dependent dehydratases and a deaminase: Radical catalysis and reactivating chaperones. *Arch. Biochem. Biophys. 544*, 40−57.

(4) Gruber, K., Puffer, B., and Kräutler, B. (2011) Vitamin B_{12}-derivatives: Enzyme cofactors and ligands of proteins and nucleic acids. *Chem. Soc. Rev. 40*, 4346−4363.

(5) Frey, P. A. (2010) Cobalamin coenzymes in enzymology. In *Comprehensive Natural Products II. Chemistry and Biology* (Mander, L., and Liu, H.-W., Eds.) Vol. 7, pp 501−546, Elsevier, Oxford, U.K.

(6) Banerjee, R. (2003) Radical carbon skeleton rearrangements: Catalysis by coenzyme B_{12}-dependent mutases. *Chem. Rev. 103*, 2083−2094.

(7) Toraya, T. (2003) Radical catalysis in coenzyme B_{12}-dependent isomerization (eliminating) reactions. *Chem. Rev. 103*, 2095−2127.

(8) Zagalak, B., Frey, P. A., Karabatsos, G. L., and Abeles, R. H. (1966) The stereochemistry of the conversion of D and L 1,2-propanediols to propionaldehyde. *J. Biol. Chem. 241*, 3028−3035.

(9) Rétey, J., Umani-Ronchi, A., and Arigoni, D. (1966) Zur Stereochemie der Propandioldehydrase-Reaktion. *Experientia 22*, 72−73.

(10) Rétey, J., Umani-Ronchi, A., Seibl, J., and Arigoni, D. (1966) Zum Mechanismus der Propandioldehydrase-Reaktion. *Experientia 22*, 502−503.

(11) Bradbeer, C. (1965) The clostridial fermentations of choline and ethanolamine. II. Requirement for a cobamide coenzyme by an ethanolamine deaminase. *J. Biol. Chem. 240*, 4675−4681.

(12) Babior, B. M., Carty, T. J., and Abeles, R. H. (1974) The mechanism of action of ethanolamine ammonia-lyase, a B_{12}-dependent enzyme. The reversible formation of 5′-deoxyadenosine from adenosylcobalamin during the catalytic process. *J. Biol. Chem. 249*, 1689−1695.

(13) Diziol, P., Haas, H., Rétey, J., Graves, S. W., and Babior, B. M. (1980) The substrate-dependent steric course of the ethanolamine ammonia-lyase reaction. *Eur. J. Biochem. 106*, 211−224.

(14) Bradbeer, C. (1965) The clostridial fermentations of choline and ethanolamine. I. Preparation and properties of cell-free extracts. *J. Biol. Chem. 240*, 4669−4674.

(15) Chang, G. W., and Chang, J. T. (1975) Evidence for the B_{12}-dependent enzyme ethanolamine deaminase in *Salmonella*. *Nature 95*, 150−151.

(16) Scarlett, F. A., and Turner, J. M. (1976) Microbial metabolism of amino alcohols. Ethanolamine catabolism mediated by coenzyme B_{12}-dependent ethanolamine ammonia-lyase in *Escherichia coli* and *Klebsiella aerogenes*. *J. Gen. Microbiol. 95*, 173−176.

(17) Wolf, J. B., and Brey, R. N. (1986) Isolation and genetic characterizations of *Bacillus megaterium* cobalamin biosynthesis-deficient mutants. *J. Bacteriol. 166*, 51−58.

(18) Roof, D. M., and Roth, J. R. (1988) Ethanolamine utilization in *Salmonella typhimurium*. *J. Bacteriol. 170*, 3855−3863.

(19) Babior, B. M. (1982) Ethanolamine ammonia-lyase. In *B_{12}* (Dolphin, D., Ed.) Vol. 2, pp 263−287, John Wiley & Sons, New York.

(20) Bandarian, V., and Reed, G. H. (1999) Ethanolamine ammonia-lyase. In *Chemistry and Biochemistry of B_{12}* (Banerjee, R., Ed.) pp 811−833, John Wiley & Sons, New York.

(21) Reed, G. H. (2004) Radical mechanisms in adenosylcobalamin-dependent enzymes. *Curr. Opin. Chem. Biol. 8*, 477−483.

(22) Wallis, O. C., Johnson, A. W., and Lappert, M. F. (1979) Studies on the subunit structure of the adenosylcobalamin-dependent enzyme ethanolamine ammonia-lyase. *FEBS Lett. 97*, 196−199.

(23) Faust, L. P., and Babior, B. M. (1992) Overexpression, purification, and some properties of the AdoCbl-dependent ethanolamine ammonia-lyase from *Salmonella typhimurium*. *Arch. Biochem. Biophys. 294*, 50−54.

(24) Akita, K., Hieda, N., Baba, N., Kawaguchi, S., Sakamoto, H., Nakanishi, Y., Yamanishi, M., Mori, K., and Toraya, T. (2010) Purification and some properties of wild-type and N-terminal-truncated ethanolamine ammonia-lyase of *Escherichia coli*. *J. Biochem. 147*, 83−93.

(25) Shibata, N., Tamagaki, H., Hieda, N., Akita, K., Komori, H., Shomura, Y., Terawaki, S., Mori, K., Yasuoka, N., Higuchi, Y., and Toraya, T. (2010) Crystal structures of ethanolamine ammonia-lyase complexed with coenzyme B_{12} analogs and substrates. *J. Biol. Chem. 285*, 26484−26493.

(26) Shibata, N., Higuchi, Y., and Toraya, T. (2011) How coenzyme B_{12}-dependent ethanolamine ammonia-lyase deals with both enantiomers of 2-amino-1-propanol as substrates: Structure-based rationalization. *Biochemistry 50*, 591−598.

(27) Sun, L., and Warncke, K. (2006) Comparative model of EutB from coenzyme B_{12}-dependent ethanolamine ammonia-lyase reveals a $\beta_8\alpha_8$, TIM-barrel fold and radical catalytic site structural features. *Proteins 64*, 308−319.

(28) Sun, L., Groover, O. A., Canfield, J. M., and Warncke, K. (2008) Critical role of arginine 160 of the EutB protein subunit for active site structure and radical catalysis in coenzyme B_{12}-dependent ethanolamine ammonia-lyase. *Biochemistry 47*, 5523−5535.

(29) Bovell, A. M., and Warncke, K. (2013) The structural model of *Salmonella typhimurium* ethanolamine ammonia-lyase directs a rational approach to the assembly of the functional $[(EutB{-}EutC)_2]_3$ oligomer from isolated subunits. *Biochemistry 52*, 1419−1428.

(30) Chen, Z.-G., Ziętek, M. A., Russell, H. J., Tait, S., Hay, S., Jones, A. R., and Scrutton, N. S. (2013) Dynamic, electrostatic model for the generation and control of high-energy radical intermediates by a coenzyme B_{12}-dependent enzyme. *ChemBioChem 14*, 1529−1533.

(31) Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.

(32) Gill, S. C., and von Hippel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem. 182*, 319−326.

(33) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680−685.

(34) Toraya, T., Watanabe, N., Ushio, K., Matsumoto, T., and Fukui, S. (1983) Ligand exchange reactions of diol dehydrase-bound

cobalamins and the effect of the nucleoside binding. *J. Biol. Chem. 258*, 9296−9301.
(35) Rétey, J. (1990) Enzymic reaction selectivity by negative catalysis or how do enzymes deal with highly reactive intermediates? *Angew. Chem., Int. Ed. 29*, 355−361.
(36) Babior, B. M., Moss, T. H., Orme-Johnson, W. H., and Beinert, H. (1974) The mechanism of action of ethanolamine ammonia-lyase, a B_{12}-dependent enzyme. The participation of paramagnetic species in the catalytic deamination of 2-aminopropanol. *J. Biol. Chem. 249*, 4537−4544.
(37) Warncke, K., Schmidt, J. C., and Ke, S.-C. (1999) 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. *J. Am. Chem. Soc. 121*, 10522−10528.
(38) Bender, G., Poyner, R. R., and Reed, G. H. (2008) Identification of the substrate radical intermediate derived from ethanolamine during catalysis by ethanolamine ammonia-lyase. *Biochemistry 47*, 11360−11366.
(39) Bandarian, V., and Reed, G. H. (2002) Analysis of the electron paramagnetic resonance spectrum of a radical intermediate in the coenzyme B_{12}-dependent ethanolamine ammonia-lyase catalyzed reaction of *S*-2-aminopropanol. *Biochemistry 41*, 8580−8588.
(40) Boas, J. F., Hicks, P. R., Pilbrow, J. R., and Smith, T. D. (1978) Interpretation of electron spin resonance spectra due to some B_{12}-dependent enzyme reactions. *J. Chem. Soc., Faraday Trans. 2 74*, 417−431.
(41) Buettner, G. R., and Coffman, R. E. (1977) EPR determination of the Co(II)-free radical magnetic geometry of the "doublet" species arising in a coenzyme B-12-enzyme reaction. *Biochim. Biophys. Acta 480*, 495−505.
(42) Schepler, K. L., Dunham, W. R., Sands, R. H., Fee, J. A., and Abeles, R. H. (1975) A physical explanation of the EPR spectrum observed during catalysis by enzymes utilizing coenzyme B_{12}. *Biochim. Biophys. Acta 397*, 510−518.
(43) Shibata, N., Masuda, J., Tobimatsu, T., Toraya, T., Suto, K., Morimoto, Y., and Yasuoka, N. (1999) A new mode of B_{12} binding and the direct participation of a potassium ion in enzyme catalysis: X-ray structure of diol dehydratase. *Structure 7*, 997−1008.
(44) Masuda, J., Shibata, N., Morimoto, Y., Toraya, T., and Yasuoka, N. (2000) How a protein generates a catalytic radical from coenzyme B_{12}: X-ray structure of a diol-dehydratase-adeninylpentylcobalamin complex. *Structure 8*, 775−788.
(45) Wetmore, S. D., Smith, D. M., Bennett, J. T., and Radom, L. (2002) Understanding the mechanism of action of B_{12}-dependent ethanolamine ammonia-lyase: Synergistic interactions at play. *J. Am. Chem. Soc. 124*, 14054−14065.
(46) Semialjac, M., and Schwartz, H. (2003) Computational study on mechanistic details of the aminoethanol rearrangement catalyzed by the vitamin B_{12}-dependent ethanolamine ammonia lyase: His and Asp/Glu acting simultaneously as catalytic auxiliaries. *J. Org. Chem. 68*, 6967−6983.
(47) Kawata, M., Kinoshita, K., Takahashi, S., Ogura, K., Komoto, N., Yamanishi, M., Tobimatsu, T., and Toraya, T. (2006) Survey of catalytic residues and essential roles of glutamate-α170 and aspartate-α335 in coenzyme B_{12}-dependent diol dehydratase. *J. Biol. Chem. 281*, 18327−18334.
(48) Kamachi, T., Toraya, T., and Yoshizawa, K. (2004) Catalytic roles of active-site amino acid residues of coenzyme B_{12}-dependent diol dehydratase: Protonation state of histidine and pull effect of glutamate. *J. Am. Chem. Soc. 126*, 16207−16216.
(49) Kamachi, T., Toraya, T., and Yoshizawa, K. (2007) Computational mutation analysis of hydrogen abstraction and radical rearrangement steps in the catalysis of coenzyme B_{12}-dependent diol dehydratase. *Chem.—Eur. J. 13*, 7864−7873.
(50) Jones, A. R., Hardman, S. J. O., Hay, S., and Scrutton, N. S. (2011) Is there a dynamic protein contribution to the substrate trigger in coenzyme B_{12}-dependent ethanolamine ammonia lyase? *Angew. Chem., Int. Ed. 50*, 10843−10846.
(51) Russell, H. J., Jones, A. R., Hay, S., Greetham, G. M., Towrie, M., and Scrutton, N. S. (2012) Protein motions are coupled to the reaction chemistry in coenzyme B_{12}-dependent ethanolamine ammonia lyase. *Angew. Chem., Int. Ed. 51*, 9306−9310.
(52) Jones, A. R., Levy, C., Hay, S., and Scrutton, N. S. (2013) Relating localized protein motions to the reaction coordinate in coenzyme B_{12}-dependent enzymes. *FEBS J. 280*, 2997−3008.
(53) Robertson, W. D., and Warncke, K. (2009) Photolysis of adenosylcobalamin and radical pair recombination in ethanolamine ammonia-lyase probed on the micro- to millisecond time scale by using time-resolved optical absorption spectroscopy. *Biochemistry 48*, 140−147.
(54) Robertson, W. D., Wang, M., and Warncke, K. (2011) Characterization of protein contributions to cobalt-carbon bond cleavage catalysis in adenosylcobalamin-dependent ethanolamine ammonia-lyase by using photolysis in the ternary complex. *J. Am. Chem. Soc. 133*, 6968−6977.
(55) Toraya, T., Honda, S., and Mori, K. (2010) Coenzyme B_{12}-dependent diol dehydratase is a potassium ion-requiring calcium metalloenzyme: Evidence that the substrate-coordinated metal ion is calcium. *Biochemistry 49*, 7210−7217.
(56) Kamachi, T., Takahata, M., Toraya, T., and Yoshizawa, K. (2009) What is the identity of the metal ions in the active sites of coenzyme B_{12}-dependent diol dehydratase? A computational mutation analysis. *J. Phys. Chem. B 113*, 8435−8438.
(57) Kamachi, T., Doitomi, K., Takahata, M., Toraya, T., and Yoshizawa, K. (2011) Catalytic roles of the metal ion in the substrate-binding site of coenzyme B_{12}-dependent diol dehydratase. *Inorg. Chem. 50*, 2944−2952.
(58) Kinoshita, K., Kawata, M., Ogura, K., Yamasaki, A., Watanabe, T., Komoto, N., Hieda, N., Yamanishi, M., Tobimatsu, T., and Toraya, T. (2008) Histidine-α143 assists 1,2-hydroxyl group migration and protects radical intermediates in coenzyme B_{12}-dependent diol dehydratase. *Biochemistry 47*, 3162−3173.