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Glutamate 2,3-aminomutase: a new member of the radical SAM superfamily of enzymes

Frank J. Ruzicka and Perry A. Frey

Department of Biochemistry, University of Wisconsin-Madison, 1710 University Avenue, Madison, Wisconsin 53726

SUMMARY

A gene *eam* in *Clostridium difficile* encodes a protein that is homologous to lysine 2,3-aminomutase (LAM) in many other species but does not have the lysyl-binding residues Asp293 and Asp330 in LAM from *Clostridium subterminale* SB4. The *C. difficile* protein has Lys and Asn, respectively, in the sequence positions of the essential Asp residues in LAM. The *C. difficile* gene has been cloned into an *E. coli* expression vector, expressed in *E. coli*, and the protein purified and characterized. The recombinant protein displays excellent activity as a glutamate 2,3-aminomutase and no activity toward L-lysine. The PLP-, iron-, and sulfide-content and ultraviolet/visible spectrum are similar to LAM, and the enzyme requires SAM and dithionite as activators, as does LAM. Freeze-quench EPR experiments in the presence of L-glutamate reveal a glutamate-based free radical in the steady state of the reaction. A number of other bacterial genomes include genes encoding proteins homologous to the glutamate 2,3-aminomutase from *C. difficile*, and four of these proteins display the activity of glutamate 2,3-aminomutase when produced in *E. coli*. All of the homologous proteins have the cysteine motif CSMYCRHC corresponding to the motif CxxxCxxC characteristic of radical SAM enzymes. It is concluded that glutamate 2,3-aminomutase from *C. difficile* is a representative of a family found in a number of bacteria. It is likely that the β -glutamate found in a few bacterial and archeal species as an osmolyte arises from the action of glutamate 2,3-aminomutase.

Aminomutases catalyze the reversible migration of an amino group between adjacent carbon atoms of a substrate. The known aminomutases are lysine 2,3-aminomutase (1–4), arginine 2,3-aminomutase (5), lysine 5,6-aminomutase (6–9), ornithine 4,5-aminomutase (10), phenylalanine 2,3-aminomutase (11,12), and tyrosine 2,3-aminomutase (13). Several aminomutases require PLP 1 and act by radical-based mechanisms initiated by the 5'-deoxyadenosyl radical originating with a metallo-coenzyme. One such coenzyme is a coordination complex of SAM with a unique [4Fe–4S] cluster in the reaction of lysine 2,3-aminomutase from *Clostridium subterminale* SB4 or *Bacillus subtilis* (1,2) and arginine 2,3-aminomutase from *Streptomyces griseochromogenes* (5). Lysine 5,6-aminomutase and ornithine 4,5-aminomutase require adenosylcobalamin and PLP (6–10). Other amino acid 2,3-aminomutases do not function by radical mechanisms and do not require PLP. Phenylalanine 2,3-aminomutase from *Taxus chinensis* and *Taxus cuspidata* (11,12) and tyrosine aminomutase from *Streptomyces globisporus* (13) employ methylidene imidazolone as the coenzyme.

The thoroughly studied lysine 2,3-aminomutase from *C. subterminale* SB4 catalyzes the interconversion of L-lysine and L- β -lysine (14,15). The chemical mechanism proceeds from

Communicating author: Perry A. Frey, Department of Biochemistry, University of Wisconsin-Madison, 1710 University Avenue, Madison, Wisconsin 53726. Tel, (608)262-0055; Fax, (608)265-2904; e-mail, frey@biochem.wisc.edu

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the initial formation of the 5'-deoxyadenosyl radical (5'-deoxyadenosine-5'-yl) through a [4Fe-4S]-dependent homolytic cleavage of the bond linking sulfur with C5' of the 5'-deoxyadenosyl moiety in SAM. The 5'-deoxyadenosyl radical abstracts the hydrogen atom from the 3-pro-*R* position of the lysyl side chain in the PLP-external aldimine of L-lysine at the active site to form 5'-deoxyadenosine and a substrate-related free radical, with the unpaired electron at lysyl-C3. Rearrangement of the lysyl-C3-radical is facilitated by the aldimine linkage to PLP and leads to migration of the nitrogen to C3 of the PLP-external aldimine of L-β-lysine, with the unpaired electron at C2. The latter is the product-related radical, which subsequently abstracts a hydrogen atom from the methyl group of 5'-deoxyadenosine into the 2-pro-*R* position of the external PLP-aldimine of L-β-lysine, and the resultant 5'-deoxyadenosyl radical regenerates SAM (14–19). Reversible, homolytic cleavage of SAM into the 5'-deoxyadenosyl radical is accomplished by way of inner sphere electron transfer between the [4Fe-4S] cluster and SAM, which is directly ligated to the iron-sulfur center (4,20,21). The x-ray crystallographic analysis of the enzyme from *C. subterminale* SB4 (4) and the EPR (17,18), ENDOR (20), ESEEM (19), and XAS (21) spectroscopic studies substantiate this mechanism.

The x-ray crystal structure of *C. subterminale* SB4 lysine 2,3-aminomutase identifies several amino acid residues that appear important for the binding of L-lysine at the active site (4). The structure shows two aspartate residues, D293 and D330, in close, apparently hydrogen bonded contacts with the ε-amino group of lysine. A search of the genomic database reveals several amino acid sequences that are homologous to that of lysine 2,3-aminomutase, but in which the amino acids corresponding to positions 293 and 330 are lysine and asparagine, not aspartate. If these genes encode an amino acid 2,3-aminomutase, the substrate is not expected to be L-lysine. The substrate might possibly be L-glutamate, in which case the enzymes could be glutamate 2,3-aminomutases.

Lysine 2,3-aminomutase has been known for more than 25 years, but no glutamate 2,3-aminomutase has been described. Methanogenic archaeobacteria accumulate high levels of β-glutamate, as well as L-glutamate and *N*(ε)-acetyl-β-lysine, which are thought to serve as osmoregulators in response to high levels of external salt (22,23). Here we show through gene cloning and expression that *Clostridium difficile* produces a highly active glutamate 2,3-aminomutase that is homologous with lysine 2,3-aminomutase.

MATERIALS AND METHODS

Materials

The following were obtained from the named vendors: Tris, carbenicillin, chloramphenicol, EPPS, PLP, PMSF, streptomycin sulfate, SAM, sodium hydrosulfite, L-glutamate, β-glutamate, and the GenElute Minus EtBr spin column from Sigma; agarose, XhoI, calf-intestine alkaline phosphatase, T4 DNA ligase buffer, and T4 DNA ligase from Promega; DNA polymerase reaction buffer, Pfu DNA polymerase, Epicurian coli XL2-Blue MRF⁺ and *E.coli* BL21(DE3) CodonPlus RILP cells from Stratagene; PCR Blunt II TOPO vector and TOP10 One Shot *E. coli* cells from Invitrogen; IPTG and DTT from Inalco; phenyl-Sepharose 6 Fast Flow (high substitution), Q-Sepharose Fast Flow, and Sephacryl S200 HR from Amersham Biosciences; ampicillin and kanamycin from Fisher; DL-[2-¹³C] glutamate and L-[2,3,3,4,4-d₅]-glutamate from Aldrich-Isotech; chromosomal DNA # 9689D from American Tissue Type Cell Collection; Qiagen Plasmid mini kit from Qiagen; NdeI from New England Biolabs; pET23a(+) and pET21a(+) from Novagen; FeSO₄ from JT Baker; PITC from Pierce; 10–20% polyacrylamide Ready Gels from Bio-Rad; and amino acid standard solution #20088 from Pierce. All other chemicals were obtained in highest available purity from commercial vendors and used as supplied.

Cloning of *eam* from *C. difficile* encoding glutamate 2,3-aminomutase

The nucleotide sequence of the gene *eam* in *C. difficile* encoding a protein with >50% amino acid sequence identity to lysine 2,3-aminomutase from *C. subterminale* SB4 (US Patent 6248874 B1) was obtained from the Sanger Institute genomic database (reverse complement of *C. difficile* nucleotide sequence – check:1725 from 887–2155). The gene was cloned from *C. difficile* chromosomal DNA by PCR techniques. Genomic DNA was dissolved in TE buffer (10 mM Tris-HCl, with 1 mM EDTA) at pH 8.0 to a concentration of 0.5 g/L. The PCR reaction mixture (100 L) contained: *C. difficile* strain 630 chromosomal DNA, 1 g; cloned Pfu DNA polymerase reaction buffer; dNTPs, 0.2 mM each; oligonucleotide primers (Primer A – 5'-ACACATATGAATGAACAACTAGAAATATCCTTAG and Primer B – 5'-TGACTCGAGTTAATCCTGTGCTTGTTCCTTTATAAT), 25 pmoles each; and cloned Pfu DNA polymerase, 5 units. All samples were overlaid with mineral oil and subjected to 30 cycles of 1 min. at 94 °C, 30 s at 37 °C, 15 s at 50 °C, and 2 min. at 72 °C. After thermocycling, DNA was further purified by agarose electrophoresis (2% agarose in TAE buffer (0.04 M Tris-acetate at pH 8.0 with 1 mM EDTA). Following identification and excision of the appropriately sized (1272 bp) ethidium bromide stained band, DNA was extracted from the agarose using the GenElute Minus EtBr spin column, concentrated by precipitation with ethanol, and resuspended in TE buffer at pH 8.0.

The purified PCR product was blunt-end ligated to PCR Blunt II TOPO vector using 0.5 L of PCR insert product to 1 L of vector according to the manufacturer's specifications. The ligated DNA was used to transform TOP10 One Shot *E. coli* cells which were subsequently plated on LB + kanamycin (50 g/mL) plates and cultured overnight. Individual colonies were chosen and subcloned in LB + kanamycin (50 g/mL) media for plasmid purification. Plasmid DNA was purified using the Qiagen Plasmid mini kit. To confirm the nucleotide sequence, the inserted gene, herein named *eam*, was sequenced in its entirety in four colonies at the University of Wisconsin Biotechnology Center.

Purified PCR Blunt II TOPO + gene insert plasmid was subjected to restriction digestion. For the gene insert, 10 g of plasmid DNA was cut with NdeI (20 units) and XhoI (10 units) in a total volume of 100 L for 1 h at 37 °C. The insert DNA was separated from the plasmid DNA by agarose gel electrophoresis (2% agarose in TAE buffer) and purified and concentrated as previously described. The expression vector, pET23a(+) was similarly cut with NdeI and XhoI, dephosphorylated at the 5'-end with calf-intestine alkaline phosphatase (1 unit) for 30 min. at 37 °C, purified by agarose gel electrophoresis, and concentrated by ethanol precipitation. The pET-23a(+) insert and the pET-23a(+) cut vector were ligated with T4 DNA ligase. To 3 ng of insert DNA were added 10 ng of cut vector in T4 DNA ligase buffer + T4 DNA ligase (3 units) in a total volume of 10 L and incubated for 16 h at 14 °C. Competent *E. coli* were transformed with 2 L ligation mix and plated on LB + carbenicillin (100 g/mL) plates and plasmid DNA isolated as described previously. The pET23a(+) gene insert was sequenced in entirety to confirm the correctness of the construct.

Expression of *eam* in *E. coli*

The pET23a(+) gene insert expression vector was transformed into competent *E. coli* BL21 (DE3) CodonPlus RILP cells. This cell line is a λ DE3 lysogen carrying the gene for T7 RNA polymerase under control of IPTG. For transformation, 100 L of competent cells were treated with 54 ng of plasmid DNA and heat shocked for 20 s at 42 °C. After transformation, 10 L of cells were plated on LB + carbenicillin (100 g/mL) + chloramphenicol (34 g/mL) plates and grown overnight at 37 °C. Individual colonies were subcultured in LB media containing carbenicillin (100 g/mL) and chloramphenicol (34 g/mL) to prepare frozen stocks. Cells were thawed and streaked on LB plates containing carbenicillin (100 g/mL) and chloramphenicol (34 g/mL). Individual colonies were grown in LB media containing carbenicillin (100 g/mL)

and chloramphenicol (34 g/mL) for approximately 6 h at 37 °C and used to inoculate 2 liter shake flasks, each of which contained 1 liter of LB medium with ampicillin (100 g/mL) and chloramphenicol (34 g/mL) and supplemented with 100 M FeCl₃. Cells were cultured overnight (16 h) at 37 °C with slow shaking (100 RPM). IPTG was added to 1 mM, and culturing continued for 4 h at 37 °C. Cells were centrifuged at $6,000 \times g_{av}$ for 10 min and, frozen in small portions in liquid N₂, and stored at -70 °C. Approximately 40 g (wet) were obtained from 10 L of culture.

Purification of Glutamate 2,3-Aminomutase

All purification steps except centrifugation were conducted in an anaerobic chamber (Coy, Grass Lake, MI) at room temperature, except as otherwise specified. For centrifugation, suspensions were sealed into centrifuge bottles inside the anaerobic chamber, removed from the chamber and centrifuged at 4 °C, returned to the chamber and unsealed.

Cells (40 g) were thawed in 70 mL standard buffer (SB) [0.03 M sodium EPPS at pH 8.0, 40 M FeSO₄, 40 M PLP, 1 mM L-glutamate, 1 mM DTT, and 1 mM PMSF] prior to sonication using the standard tip of the Sonic Dismembrator (Misonix, Farmingdale, NY, Model #3000) (setting 8 for six 30 sec. intervals at 4–8 °C). Following sonication, cells were centrifuged at $20,000 \times g_{av}$ for 30 min. The supernatant fluid was subjected to streptomycin sulfate treatment (3% at 4 °C) to remove nucleic acids, and then to ammonium sulfate treatment (70% saturation at 4 °C) to precipitate proteins. Each precipitation was followed by centrifugation at $20,000 \times g_{av}$ for 20 min at 4 °C.

The protein was dissolved in SB + 1 M ammonium sulfate and applied to a phenyl-Sepharose 6 Fast Flow (high substitution) column (2.5 × 25 cm) previously equilibrated with the same buffer. The adsorbed enzyme was eluted with a linear gradient formed from 2 L of SB + 1 M ammonium sulfate and 2 L of SB. The gradient started at 1 M ammonium sulfate and decreased linearly at 2.5 mL/min. Glutamate 2,3-aminomutase emerged near the end as a yellow-brown band. Desired fractions were pooled, the protein precipitated by addition of solid ammonium sulfate (70% saturation 4°C) and centrifuged. The protein was redissolved in 750 mL of SB and applied to a Q-Sepharose Fast Flow column (2.5 × 25 cm) previously equilibrated with SB. Protein was eluted with a linear gradient formed from 2 L of SB and 2 L of SB + 0.5 M NaCl at 2.5 mL/min. Glutamate 2,3-aminomutase emerged in the latter half of the gradient as a yellow-brown band that was collected and concentrated by ammonium sulfate precipitation (70% saturation at 4 °C) and centrifugation at $20,000 \times g_{av}$ for 20 min. The pellet was dissolved in 6 mL of SB buffer and passed through a Sephacryl S200 HR column (2.5 × 25 cm) equilibrated with SB at a flow rate of 2 mL/min. The protein was collected and concentrated using Centricon YM10 (Amicon, Bedford, MA) spin concentrators to a concentration of 20 mg/mL, frozen as droplets in liquid N₂, and stored in liquid N₂.

Quantitative Amino Acid Analysis

The concentration of purified *C. difficile* glutamate 2,3-aminomutase was based on the known amino acid composition and measured by quantitative amino acid analysis. Aliquots of enzyme (5 L) were added to hydrolysis tubes (Pierce Chemical Co.), each containing 700 L of 6N HCl. To half the tubes, 25 L of amino acid standard solution (2.5 mM of each acid) was added. Tubes were sealed *in vacuo* and heated for 24, 48, and 72 h at 110 °C. Following hydrolysis, samples were transferred to 2 mL microcentrifuge tubes and concentrated to dryness by vacuum centrifugation. The dried samples, with and without amino acid standards, were derivatized with PITC and separated by HPLC as described (24) by dissolution in 100 L of coupling buffer, concentration to dryness by vacuum centrifugation, redissolution in 100 L coupling buffer plus 10 L of PITC, reaction for 30 min at room temperature, concentration to dryness, and dissolution in 1 mL of water prior to separation in a Beckman Gold HPLC. The PITC

derivatives (50 L) were separated on a C18 column (Phenomenex Gemini 5 4.6 mm × 25 cm) at room temperature and a flow rate of 1 mL/min. A complex linear gradient starting with 0.05 M ammonium acetate in water (A) and 0.10 M ammonium acetate in 44% water, 46% acetonitrile, and 10% methanol (B) separated the PITC-amino acids. The gradient established was 0% B in 5 min, 0–10% B in 40 min, 10–35% B in 35 min, 35–55% B in 35 min, 55–100% B in 10 min. The PITC-amino acids were detected at 254 nm and emerged at the following times in min: Asx, 13; Glx, 18; Ser, 36; Gly, 39; His, 54; Thr, 56; Ala, 58; Arg, 60; Pro, 62; Tyr, 84; Val, 85; Met, 89; Ile, 98; Leu, 99; Phe, 108; Lys, 117. Each amino acid was quantitated by correlation with the added standard and used to calculate the enzyme concentration based on the known content. The enzyme concentration derived from all the amino acids were in agreement ($\pm 5\%$), and the average value was used to calculate stoichiometries from the results of analyses for iron, sulfide, and PLP, as well as the extinction coefficient for the enzyme at a given wavelength.

PLP, Iron, and Sulfide Analysis

A solution of purified glutamate 2,3-aminomutase of known micromolar concentration based on quantitative amino acid analysis, was subjected to analysis for iron, sulfide, and PLP. Iron was measured by the method of Kennedy et al (25), sulfide by the method of Beinert (26), and PLP by the method of Wada and Snell (27).

SDS PAGE Electrophoresis

SDS polyacrylamide gel electrophoresis was conducted according to the method of Laemmli (28) using Tris-glycine buffer {0.025M Tris, 0.19 M glycine, 0.1% SDS (sodium dodecyl sulfate)} and 10–20% polyacrylamide Ready Gels.

Assay of Glutamate 2,3-Aminomutase Activity

Enzyme assays were conducted at room temperature within an anaerobic chamber (Coy, Grass Lake, MI) at 37 °C. Glutamate 2,3-aminomutase (0.15 M subunits) was mixed with the following: Na-EPPS, 60 mM at pH 8.0; SAM, 250 M; sodium hydrosulfite, 65 M; L-glutamate or β -glutamate, 20 mM. At timed intervals (0–3 min), 45 L of reaction mixture was quenched with 15 L of 2N perchloric acid. Samples were centrifuged at 14,000×g for 10 min and supernatant fluids treated with PITC by the following method (24). Samples were dissolved in 100 L of coupling buffer (24), concentrated to dryness by vacuum centrifugation, redissolved in 400 L coupling buffer plus 20 L of PITC, set aside to react for 30 min, concentrated to dryness as before, and dissolved in water (0.5 or 1.0 mL) prior to HPLC. The PITC-derivatives of L-glutamate and β -glutamate ((50 or 100 L) were separated (24) using a C18 column (Phenomenex Gemini 5, 4.6 mm × 25 cm, #G-4435-E0), flow rate 1 mL/min. Separation was with a linear gradient formed with 0.05 M ammonium acetate in water (A) and 0.1 M ammonium acetate in 44% water, 46% acetonitrile, and 10% methanol (B). The gradient was 0–10% B in 20 min, 10–100% B in 10 min. PITC-L-glutamate emerged at 14.8 – 15.0 min and PITC- β -glutamate at 15.5 – 15.7 min. PITC-derivatives of L-glutamate and β -glutamate in quenched samples were measured by reference to amino acid standards. The concentrations of L-glutamate and β -glutamate in quenched samples were determined from the fractions of the PITC-derivatives in the HPLC analysis. The peak area of either PITC-L-glutamate or PITC- β -glutamate was divided by the sum of the two and multiplied by the starting concentration of either L-glutamate or β -glutamate to obtain molar concentrations. Initial rates were determined at 0.6 – 20 mM L-glutamate and used to calculate kinetic parameters ($V_{\max}$ and K_m) by the nonlinear least squares curve fitting method of Cleland (29). Parameters determined 0.75 and 0.15 M enzyme were in agreement.

Equilibrium Constant

Either L-glutamate or β -glutamate at 0.6 to 20 mM were placed with glutamate 2,3-aminomutase and required cofactors at initial enzyme concentrations of 0.75 M or 0.15 M in EPPS buffer at pH 8.0 and 24 °C. After approximately 4 h, an additional aliquot of enzyme was added to make the final enzyme concentration either 1.5 M or 0.3 M, and the reaction was allowed to proceed for an additional 16 h. The reaction was stopped with 0.55 N perchloric acid, and the amino acids were derivatized with PITC as previously described. Following HPLC separation of the PITC derivatized L-glutamate and β -glutamate, the ratio of peak areas was calculated based on the equivalent extinction coefficient of the two compounds (24).

EPR Spectroscopy

Samples of enzyme as isolated (180 M in subunits) were quickly mixed anaerobically at 24 °C with Tris-sulfate at 80 mM and pH 8.0, SAM at 1 mM, sodium dithionite at 1 mM and one of the following substrates: A) L-glutamate at 50 mM; B) β -glutamate at 50 mM; C) DL-[2-¹³C]glutamate at 100 mM; D) L-[2,3,3,4,4-d₅] glutamate at 50 mM; E) L-glutamate 50 mM with SAM omitted; F) L-Glutamate at 50 mM with sodium dithionite omitted. The samples were frozen at -150 °C as soon as possible after mixing (total time < 30 sec) The EPR spectra of the frozen samples were recorded at 77 °K using a Varian model E3 spectrometer with the following settings: Field center 3260 Gauss; Scan width 200 Gauss; Microwave frequency 9.1 GHz; Microwave power 5 milliWatts; Modulation frequency 100 kHz; Modulation amplitude 2.0 Gauss; Time constant 0.3 sec; Scan time 240 sec. Gain 80,000 (A,B,E, F), 320,000 (C), 40,000 (D).

UV-Visible Absorption Spectrum

The UV-Visible absorption spectrum of the purified enzyme (0.45 mg/mL) in 0.02M sodium EPPS buffer at pH 8.0 was measured anaerobically with a Hewlett-Packard Diode Array Spectrophotometer (Model 8452A).

D293K/D330N-Lysine 2,3-aminomutase

The double mutant of *C. subterminale* SB4 lysine 2,3-aminomutase was prepared by site-directed mutagenesis of the *kamA* gene (30) cloned in the pTrc99A vector for expression. The procedure utilized the Quikchange site-directed mutagenesis kit by Stratagene following recommended procedures and utilizing the following mutagenic primers: for D293K – Primer A – 5'-CTACATCTATCAATGTAAGTTATCATTAGGACTTG, Primer B – 5'-CAAGTCCTAATGATAACTTACATTGATAGATGTAG; for D330N – Primer A – 5'-CCAACATTTCGTTGTTAACGCTCCAGGTGGTGGTG, Prime B – 5'-CACCACCACCTGGAGCGTTAACACGAATGTTGG. The mutated gene was sequenced to confirm the mutated sites prior to expression in BL21 Gold Codon Plus RIL *E. coli* cells (Stratagene). The enzyme was produced, and purified as described elsewhere (30). The purified variant did not display either lysine or glutamate 2,3-aminomutase activity.

Glutamate 2,3-aminomutase from *Thermoanaerobacter tengcongensis* MB4 and *Syntrophomonas wolfei* str. Goettingen

Synthetic genes for *Thermoanaerobacter tengcongensis* MB4 gene (NC 003869) and *Syntrophomonas wolfei* str. Goettingen gene (NZ AAJG01000011) optimized for expression in *E. coli* were obtained from Genscript Corp. The synthetic genes contained restriction sites NdeI and XhoI permitting cloning into pET21a(+) expression vector and expression in BL21 (DE3) *E. coli* cells as previously described for *eam* (See Cloning and Expression of *eam*). Cell extracts of each were prepared following sonication and centrifugation (See Purification of Glutamate 2,3-aminomutase). Extracts were measured for glutamate 2,3-aminomutase (See Assay of Glutamate 2,3-aminomutase Activity).

RESULTS

Homologs of Lysine 2,3-Aminomutase

More than 55 apparent species variants of lysine 2,3-aminomutase can be found in the bacterial genomic databases (30). Nearly all are lysine 2,3-aminomutases; however, a few display two important differences in amino acid sequence from the lysine 2,3-aminomutases. The amino acid sequences of the latter variants are in Fig. 1, aligned with that of lysine 2,3-aminomutase from *C. subterminale* SB4 shown at the bottom. Key active site residues include the cysteine-cluster CxMxCrxC corresponding to the motif CxxxCxxC characteristic of radical SAM enzymes (33). The iron-binding cysteine-motif in lysine 2,3-aminomutase includes Cys125, Cys129, and Cys132 and is identified in the lower sequence. Lysine-binding residues in lysine aminomutase are Arg134, which binds the α -carboxylate group, and Asp293 and Asp330, which bind the ϵ -aminium group (4). Lys337 binds PLP. These key residues are identified in Fig. 1 and enclosed within boxes with the corresponding aligned residues in the other species. Strictly conserved residues in all of the sequences are shown in red. The ninety-eight strictly conserved amino acids in the aligned sequences clearly document the homologous relationship among the proteins. The striking differences in the upper sequences are the presence of Lys and Asn in place of Asp293 and Asp330 in lysine 2,3-aminomutase. It is clear that Lys and Asn could not bind the lysyl side chain. The conservation of Arg134 is compatible with an amino acid substrate; in this case the substrate might be an acidic amino acid such as glutamate, and a glutamate 2,3-aminomutase could explain the presence of β -glutamate in certain bacterial species.

The Aminomutase-Homologous Protein in *C. difficile*

To determine whether the lysine 2,3-aminomutase-homologous enzymes in Fig. 1 might be glutamate 2,3-aminomutases, the gene encoding this enzyme in *C. difficile*, herein named *eam*, was cloned into the *E. coli* plasmid expression vector, pET23a(+). Transformation of this plasmid into *E. coli* led to high level production of the protein in cells (Fig. 2). As shown in lane 2, SDS PAGE of a cellular extract from *E. coli* cells following induction indicated a dominant band at approximately 50 kDa, with many minor bands for other cellular proteins. The size of the major protein is comparable to the calculated molecular weight of 48.9 kDa for the gene product based on the amino acid composition. Purification of the recombinant protein by a conventional column chromatography procedure based on that for lysine 2,3-aminomutase yielded approximately 200 mg of purified protein from 40 g (wet weight) of *E. coli* cells. Based on lane 3 of Fig. 2, the purified protein is estimated to be 95% pure. The high purity of the recombinant protein is supported by the amino acid analysis.

Amino acid analysis

Quantitative amino acid analysis allowed for an accurate measurement of the protein concentration in solutions, and this facilitated measurements of cofactor content, kinetic parameters, and extinction coefficients. The content of amino acids other than Ser, Thr, Asx, Cys, and Trp were used to measure the protein concentration following complete acid hydrolysis and amino acid analysis by an HPLC procedure. Ser and Thr content decreased with extended hydrolysis as expected (31). Analysis for Asx (Asp + Asn) increased at 48 and 72 hours hydrolysis due to the partial conversion of cysteine to cysteic acid, which is not distinguished from Asp in the chromatograms. The analysis for amino acids was indistinguishable ($\pm 5\%$) from the amino acid content calculated from the sequence in Fig. 1 for the protein in *C. difficile*.

Cofactor content and physical parameters

Based on the quantitative amino acid analysis, the stoichiometric content of cofactors, the kinetic parameters, and the extinction coefficients for the recombinant protein could be accurately measured. As shown in Fig 3, the recombinant protein displays activity as a glutamate 2,3-aminomutase. PITC-derivatized L-glutamate and β -glutamate are separated by HPLC. Fig. 3A shows HPLC chromatograms demonstrating the conversion of L-glutamate to β -glutamate in solutions containing the purified protein. Shown in Fig. 3B is the transformation of β -glutamate into L-glutamate. Both reactions require the presence of SAM and dithionite, as in the action of lysine 2,3-aminomutase. The two reactions, starting with either L-glutamate or β -glutamate as substrate, led to the same equilibrium mixture, with the equilibrium constant favoring β -glutamate, as shown in Table 1.

The PITC-based HPLC assay was employed to measure the kinetic parameters. In the standard assays, conducted at 20 mM L-glutamate or β -glutamate in the forward and reverse directions, the enzyme catalyzes production of β -glutamate at a rate of $5.9 \text{ moles min}^{-1} \cdot (\text{mg protein})^{-1}$, and in the reverse direction it produces L-glutamate at a rate of $2.3 \text{ moles min}^{-1} \cdot (\text{mg protein})^{-1}$. The turnover numbers for the interconversion of L-glutamate and β -glutamate are within an order of magnitude of the values for lysine 2,3-aminomutase, and the values of K_m are also similar in the millimolar range, as set forth in Table 1.

The purified enzyme contains iron, sulfide and PLP in the stoichiometric amounts given in Table 1. The PLP-content corresponds very well to one molecule per subunit. The iron and sulfide contents are similar to each other and less than the four per subunit that would be expected for one $[4\text{Fe-4S}]$ cluster per subunit. The iron and sulfide stoichiometries are similar to those for lysine 2,3-aminomutase, as purified by chromatography and not reconstituted with iron and sulfide, some of which is lost during purification. Presumably, the iron-sulfur cluster in glutamate 2,3-aminomutase is also labile to chromatography, which would account for the low iron and sulfide content. Perhaps the iron and sulfide content of glutamate 2,3-aminomutase would be increased by reconstitution, as it is in the case of lysine 2,3-aminomutase (30); however, reconstitution has not been attempted.

The ultraviolet/visible absorption spectrum of glutamate 2,3-aminomutase from *C. difficile* resembles that of lysine 2,3-aminomutase from *C. subterminale* SB4 (30). The spectrum of the purified enzyme displays prominent peaks at 280 and 420 nm typical of absorption due to amino acid components (Trp and Tyr) at 280 nm and the internal aldimine of PLP at 420 nm. Furthermore a broad overlapping absorption band is observed decreasing from 300 nm to 700 nm, a characteristic of proteins containing $[4\text{Fe-4S}]$ clusters. The extinction coefficients of the purified enzyme at the two wavelength-maxima are included in Table 1. The preferred method for measuring the concentration of this protein is to measure A_{280} and calculate the concentration from Beer's Law and the extinction coefficient.

Observation of a radical intermediate

EPR spectroscopy of the purified enzyme in the presence of either L-glutamate or β -glutamate and SAM and dithionite (Fig. 5) reveals the presence of a free radical analogous to the product-related radical reported in the reaction of *C. subterminale* SB4 lysine 2,3-aminomutase with L-lysine (17). The splittings in the EPR signal result from nuclear hyperfine coupling of the C2(H) with the unpaired electron on C2. Figure 5D shows that the use of $[2,3,3,4,4\text{-d}_5]$ -L-glutamate in place of L-glutamate leads to the collapse of the signal into a broadened singlet. This arises from the 6-fold smaller splitting constant for deuterium relative to hydrogen. The spectrum shown in Fig. 6C proves that the EPR signal originates with a radical in which the unpaired electron is centered on C2 of the substrate-carbon skeleton. Figure 5C shows significant signal broadening and alterations in the splitting pattern when L- $[2\text{-}^{13}\text{C}]$ glutamate

is substituted for L-glutamate. In the absence of added SAM or dithionite, no EPR signal can be observed (Fig. 5E,F).

In vivo activity in *E. coli*

Both lysine- and glutamate-specific 2,3-aminomutases are sensitive to molecular oxygen, which oxidizes the $[4\text{Fe-4S}]^{1+}$ cluster required for catalysis to $[4\text{Fe-4S}]^{2+}$. Molecular oxygen further oxidizes the cluster, with degradation to a three-iron cluster and lower forms (32). When produced in *E. coli* grown aerobically in shake flasks by heterologous expression of genes from *Clostridia*, both enzymes are active. In the case of glutamate 2,3-aminomutase in *E. coli* grown in aerobic shake flasks, analysis of the protein-free cell extract shows the presence of both L-glutamate and β -glutamate, predominantly β -glutamate. It appears that the reaction *in vivo* is near equilibrium, in accord with the equilibrium constant in Table 1.

Expression in *E. coli* of synthetic genes encoding the homologous proteins from two other species, either *Thermoanaerobacter tengcongensis* or *Syntrophomonas wolfei* str. *Goettingen*, also produces glutamate 2,3-aminomutase activity in cell extracts. These species of glutamate 2,3-aminomutase have not been fully characterized but are likely to be similar to the enzyme from *C. difficile*, based on similarity of amino acid sequences.

DISCUSSION

The PLP, iron, and sulfide content of glutamate 2,3-aminomutase and requirement for SAM and dithionite are the same as those of lysine 2,3-aminomutase, and the amino acid sequences of the two enzymes are similar. On this basis, the two enzymes are likely to act by similar chemical mechanisms, while displaying different substrate specificities. In support of this proposition, the free radical observed by EPR spectroscopy in the steady state of the action of glutamate 2,3-aminomutase in the present work is analogous to that observed in the reaction of lysine 2,3-aminomutase (17). The two free radicals display spin on C2 of the carbon skeletons of the respective substrates. The two free radicals are the product-related radicals in the radical isomerization mechanism established for the action of lysine 2,3-aminomutase. This mechanism can be adapted to the reaction of glutamate 2,3-aminomutase, as shown in Figure 6. The mechanism is the same as for lysine 2,3-aminomutase, with the substitution of a carboxymethyl group ($^-\text{OOC-CH}_2^-$) in L-glutamate for the aminopropyl group ($^+\text{H}_3\text{N-CH}_2\text{CH}_2\text{CH}_2^-$) in L-lysine. The EPR spectrum of the free radical observed in the steady state of the reaction of glutamate 2,3-aminomutase in Figure 5 is radical **3** in Figure 6.

The chemical mechanism in Figure 6 is initiated by the reversible cleavage of SAM into the 5-deoxyadenosyl radical and methionine at the active site of glutamate 2,3-aminomutase. This is likely brought about by electron transfer from the $[4\text{Fe-4S}]^{1+}$ cluster by the same mechanism as in the action of lysine 2,3-aminomutase (14–21).

Based on the structure of lysine 2,3-aminomutase with lysine bound to the active site (4) and the comparative amino acid sequences of the two enzymes, the binding of glutamate to the active site of glutamate 2,3-aminomutase can be expected to involve Arg173, Lys332, and Asn369 as illustrated in Fig. 7. Shown are the electrostatic and hydrogen bonded interactions of lysine with lysine 2,3-aminomutase (4) and the proposed corresponding interactions of glutamate with glutamate 2,3-aminomutase. The two enzymes are proposed to bind the α -carboxylate groups of the respective substrates similarly with Arg134 and Arg173. The differences are in the binding of the substrate-side chains. Asp293 and Asp330 bind the ϵ -aminium group of lysine in lysine 2,3-aminomutase; whereas, Lys332 and Asn369 are proposed to bind the γ -carboxylate group of glutamate in glutamate 2,3-aminomutase.

While the foregoing proposal for binding glutamate to glutamate 2,3-aminomutase is reasonable and likely to be correct in essential respects, the binding of glutamate and its relationship to catalysis is much more complex and not explained by the simple picture in Fig. 7. This is known because the mutation in lysine 2,3-aminomutase of Asp293 to Lys and of Asp330 to Asn, that is to the doubly mutated D293K/D330N-variant, does not lead to activity as a glutamate 2,3-aminomutase (see MATERIALS AND METHODS). Other differences between lysine and glutamate 2,3-aminomutases must exist. We note that the double mutation D293K/D330N in the variant leads to a substantial change in the electrostatic charge in the active site of the free enzyme. Presumably, secondary structural consequences of this change must be compensated in the true glutamate 2,3-aminomutase.

β -Glutamate is reported to serve as an osmolyte in certain bacteria and archaea (22,23). The origin of β -glutamate has not been known in the past. The most obvious source would be through the action of a glutamate 2,3-aminomutase. As illustrated in Fig. 1 and shown by the experiments reported herein, glutamate 2,3-aminomutases are widespread in bacterial and archaeal species and are likely to generate β -glutamate in those species in which it serves as an osmolyte.

A possible additional function for glutamate 2,3-aminomutase might be found in the metabolism of glutamate. Three pathways for glutamate breakdown in anaerobes are known (34). In two lengthy pathways, L-glutamate is first transformed into β -methylaspartate by glutamate mutase, and the latter is further metabolized to pyruvate and acetate. In a third lengthy pathway, L-glutamate is transformed into ammonia and 2-hydroxyglutaryl-CoA, dehydrated to glutaconyl-CoA, and decarboxylated to carbon dioxide and crotonyl-CoA. A fourth, shorter pathway can be envisioned starting with glutamate 2,3-aminomutase. Transformation of L-glutamate into β -glutamate, thence to ammonia and 3-oxoglutarate could produce acetoacetate, following decarboxylation of 3-oxoglutarate. Acetoacetate is straightforwardly converted into two moles of acetyl-CoA or acetate. It is interesting that all four pathways require steps that proceed by radical mechanisms, the steps catalyzed by glutamate mutase (34), glutaconyl-CoA dehydratase (34) and, as shown in the present work, glutamate 2,3-aminomutase. There is currently no evidence for the fourth or any other hypothetical pathway requiring glutamate 2,3-aminomutase.

Enzymes of the radical SAM superfamily catalyze diverse biochemical reactions (33). Examples include, in addition to lysine 2,3-aminomutase, pyruvate formate lyase activase and anaerobic ribonucleotide reductase activase, which catalyze glycyl radical formation in glycyl radical enzymes (35-38); biotin synthase and lipoyl synthase, which catalyze sulfur insertion in the biosyntheses of biotin and lipoic acid, respectively (39-41); coproporphyrinogen oxidase, which catalyzes the oxidative decarboxylation of the propionate side chains to vinyl groups in the biosynthesis of heme in anaerobes (41); MoaA, which participates in catalyzing the biosynthesis of molybdopterin (42); and spore photoproduct lyase, which repairs methylene-bridged thymine dimers in DNA (43,44). Crystal structures of four of these enzymes are available (4,42,45,46).

The equilibrium in the interconversion of L-glutamate and β -glutamate strongly favors the beta-amino acid (Table 1), as does the interconversion of L-lysine and L- β -lysine. The accompanying paper shows that the equilibrium constant favoring L- β -lysine can be attributed to the greater enthalpic stability of L- β -lysine, most likely to the greater strength of the β -C—N bond relative to the α -C—N bond (47). The same effect can account for the equilibrium constant in the reaction of glutamate 2,3-aminomutase.

All of the radical SAM enzymes studied to date employ the unique [4Fe-4S]¹⁺ cluster nucleated by the CxxxCxxC motif in the reductive cleavage of S-adenosylmethionine to generate the 5'-

deoxyadenosyl radical, which in turn initiates the radical mechanisms. The radical SAM superfamily includes many other enzymes that have not been studied (33). Glutamate 2,3-aminomutase can now be added to the rapidly growing list of radical SAM enzymes.

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References

1. Frey PA. P.A. Radical mechanisms of enzymatic catalysis. *Annu Rev Biochem* 2001;70:121–148. [PubMed: 11395404]
2. Frey PA, Magnusson OTh. S-Adenosylmethionine: a wolf in sheep's clothing, or a rich man's adenosylcobalamin? *Chem Rev* 2003;103:2129–2148. [PubMed: 12797826]
3. Chen D, Ruzicka FJ, Frey PA. A novel lysine 2,3-aminomutase encoded by the *yodO* gene of *Bacillus subtilis*: characterization and the observation of organic radical intermediates. *Biochem J* 2000;348:539–549. [PubMed: 10839984]
4. Lepore BW, Ruzicka FJ, Frey PA, Ringe D. The x-ray crystal structure of lysine-2,3-aminomutase from *Clostridium subterminale*. *Proc Natl Acad Sci U S A* 2005;102:13819–13824. [PubMed: 16166264]
5. Prabhakaran PC, Woo NT, Yorgey PS, Gould SJ. Biosynthesis of Blasticidin S from L-alpha-arginine. Stereochemistry in the arginine 2,3-aminomutase reaction. *J Am Chem Soc* 1988;110:5785–5791.
6. Chang CH, Frey PA. Cloning, sequencing, heterologous expression, purification, and characterization of adenosylcobalamin-dependent D-lysine 5, 6-aminomutase from *Clostridium sticklandii*. *J Biol Chem* 2000;275:106–114. [PubMed: 10617592]
7. Tang KH, Chang CH, Frey PA. Electron transfer in the substrate-dependent suicide inactivation of lysine 5,6-aminomutase. *Biochemistry* 2001;40:5190–5199. [PubMed: 11318641]
8. Tang KH, Harms A, Frey PA. Identification of a novel pyridoxal 5'-phosphate binding site in adenosylcobalamin-dependent lysine 5,6-aminomutase from *Porphyromonas gingivalis*. *Biochemistry* 2002;41:8767–8776. [PubMed: 12093296]
9. Berkovitch F, Behshad E, Tang KH, Enns EA, Frey PA, Drennan CL. A locking mechanism preventing radical damage in the absence of substrate, as revealed by the x-ray structure of lysine 5,6-aminomutase. *Proc Natl Acad Sci U S A* 2004;101:15870–15875. [PubMed: 15514022]
10. Chen HP, Wu SH, Lin YL, Chen CM, Tsay SS. Cloning, sequencing, heterologous expression, purification, and characterization of adenosylcobalamin-dependent D-ornithine aminomutase from *Clostridium sticklandii*. *J Biol Chem* 2001;276:44744–44750. [PubMed: 11577113]
11. Steele, CL.; Chen, Y.; Dougherty, BA.; Hofstead, S.; Lam, KS.; Li, W.; Xing, Z. US Patent AN 2003. 633941. 2003.
12. Walker KD, Klettke K, Akiyama T, Croteau R. Cloning, heterologous expression, and characterization of a phenylalanine aminomutase involved in Taxol biosynthesis. *J Biol Chem* 2004;279:53947–53954. [PubMed: 15494399]
13. Christenson SD, Liu W, Toney MD, Shen B. A novel 4-methylideneimidazole-5-one-containing tyrosine aminomutase in enediyne antitumor antibiotic C-1027 biosynthesis. *J Am Chem Soc* 2003;125:6062–6063. [PubMed: 12785829]
14. Moss M, Frey PA. The role of S-adenosylmethionine in the lysine 2,3-aminomutase reaction. *J BiolChem* 1987;262:14859–14862.
15. Baraniak J, Moss ML, Frey PA. Lysine 2,3-aminomutase. Support for a mechanism of hydrogen transfer involving S-adenosylmethionine. *J BiolChem* 1989;264:1357–1360.
16. Lieder KW, Booker S, Ruzicka FJ, Beinert H, Reed GH, Frey PA. S-Adenosylmethionine-dependent reduction of lysine 2,3-aminomutase and observation of the catalytically functional iron-sulfur centers by electron paramagnetic resonance. *Biochemistry* 1998;37:2578–2585. [PubMed: 9485408]
17. Ballinger MD, Frey PA, Reed GH. Structure of a substrate radical intermediate in the reaction of lysine 2,3-aminomutase. *Biochemistry* 1992;31:10782–10789. [PubMed: 1329955]

18. Magnusson, OTh; Reed, GH.; Frey, PA. Characterization of an allylic analogue of the 5'-deoxyadenosyl radical: an intermediate in the reaction of lysine 2,3-aminomutase. *Biochemistry* 2001;40:7773–7782. [PubMed: 11425303]
19. Ballinger MD, Frey PA, Reed GH, LoBrutto R. Pulsed electron paramagnetic resonance studies of the lysine 2,3-aminomutase substrate radical: evidence for participation of pyridoxal 5'-phosphate in a radical rearrangement. *Biochemistry* 1995;34:10086–10093. [PubMed: 7632680]
20. Chen D, Walsby CJ, Hoffman BM, Frey PA. Coordination and mechanism of reversible cleavage of S-adenosylmethionine by the [4Fe-4S] center in lysine 2,3-aminomutase. *J Am Chem Soc* 2003;125:11788–11789. [PubMed: 14505379]
21. Coper NJ, Booker SJ, Ruzicka FJ, Frey PA, Scott RA. Direct FeS cluster involvement in generation of a radical in lysine 2,3-aminomutase. *Biochemistry* 2000;39:15668–15673. [PubMed: 11123891]
22. Robertson DE, Noll D, Roberts MF. Free amino acid dynamics in marine methanogens. beta-Amino acids as compatible solutes. *J Biol Chem* 1992;267:14893–14901. [PubMed: 1353078]
23. Sowers KR, Robertson DE, Noll D, Gunsalus RP, Roberts MF. N(ε)-acetyl-beta-lysine: an osmolyte synthesized by methanogenic archaeobacteria. *Proc Natl Acad Sci USA* 1990;87:9083–9087. [PubMed: 2123548]
24. Heinrikson RL, Meredith SC. Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Anal Biochem* 1984;136:65–74. [PubMed: 6711815]
25. Kennedy MC, Kent TA, Emptage M, Merkle H, Beinert H, Munck E. Evidence for the formation of a linear [3Fe-4S] cluster in partially unfolded aconitase. *J Biol Chem* 1984;259:14463–14471. [PubMed: 6094558]
26. Beinert H. Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. *Anal Biochem* 1983;131:373–378. [PubMed: 6614472]
27. Wada H, Snell EE. The enzymatic oxidation of pyridoxine and pyridoxamine phosphates. *J Biol Chem* 1961;236:2089–2095. [PubMed: 13782387]
28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–682. [PubMed: 5432063]
29. Cleland WW. Statistical analysis of enzyme kinetic data. *Methods Enzymol* 1979;63:103–138. [PubMed: 502857]
30. Chen D, Lepore BM, Ringe D, Frey PA, Ruzicka FJ. Identification of structural and catalytic classes of highly conserved amino acid residues in lysine 2,3-aminomutase. *Biochemistry*. In press
31. Ozols J. Amino acid analysis. *Methods Enzymol* 1990;182:587–600. [PubMed: 2314259]
32. Petrovich RM, Ruzicka FJ, Reed GH, Frey PA. Characterization of iron-sulfur clusters in lysine 2,3-aminomutase by electron paramagnetic resonance spectroscopy. *Biochemistry* 1992;31:10774–10781. [PubMed: 1329954]
33. Sofia HJ, Chen G, Hetzler BG, Reyes-Spindola JF, Miller NE. Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res* 2001;29:1097–106. [PubMed: 11222759]
34. Buckel W. Unusual enzymes involved in five pathways of glutamate fermentation. *Appl Microbiol Biotechnol* 2001;57:263–273. [PubMed: 11759672]
35. Knappe J, Neugebauer FA, Blaschkowski HP, Ganzler M. Post-translational activation introduces a free radical into pyruvate formate-lyase. *Proc Natl Acad Sci U S A* 1984;81:1332–1335. [PubMed: 6369325]
36. Broderick JB, Duderstadt RE, Fernandez DC, Wojtuszewski K, Henshaw TF, Johnson MK. Pyruvate formate-lyase activating enzyme is an iron-sulfur protein. *J Am Chem Soc* 1997;119:7396–7397.
37. Eliasson R, Pontis E, Fontecave M, Gerez C, Harder J, Jornvall H, Krook M, Reichard P. Characterization of components of the anaerobic ribonucleotide reductase system from *Escherichia coli*. *J Biol Chem* 1992;267:25541–25547. [PubMed: 1460049]
38. Harder J, Eliasson R, Pontis E, Ballinger MD, Reichard P. Activation of the anaerobic ribonucleotide reductase from *Escherichia coli* by S-adenosylmethionine. *J Biol Chem* 1992;267:25548–25552. [PubMed: 1460050]

39. Sanyal I, Cohen G, Flint DH. Biotin synthase: purification, characterization as a [2Fe-2S]cluster protein, and in vitro activity of the *Escherichia coli* *bioB* gene product. *Biochemistry* 1994;33:3625–3631. [PubMed: 8142361]
40. Florentin D, Bui BT, Marquet A, Ohshiro T, Izumi Y. On the mechanism of biotin synthase of *Bacillus sphaericus*. *C R Acad Sci III* 1994;317:485–488. [PubMed: 7987699]
41. Miller JR, Busby RW, Jordan SW, Cheek J, Henshaw TF, Ashley GW, Broderick JB, Cronan JE Jr, Marletta MA. *Escherichia coli* LipA is a lipoyl synthase: in vitro biosynthesis of lipoylated pyruvate dehydrogenase complex from octanoyl-acyl carrier protein. *Biochemistry* 2000;39:15166–15178. [PubMed: 11106496]
42. Layer G, Moser J, Heinz DW, Jahn D, Schubert WD. Crystal structure of coproporphyrinogen III oxidase reveals cofactor geometry of Radical SAM enzymes. *EMBO J* 2003;22:6214–6224. [PubMed: 14633981]
43. Cheek J, Broderick JB. Direct H atom abstraction from spore photoproduct C-6 initiates DNA repair in the reaction catalyzed by spore photoproduct lyase: evidence for a reversibly generated adenosyl radical intermediate. *J Am Chem Soc* 2002;124:2860–2861. [PubMed: 11902862]
44. Buis JM, Cheek J, Kalliri E, Broderick JB. Characterization of an active spore photoproduct lyase, a DNA repair enzyme in the radical S-adenosylmethionine superfamily. *J Biol Chem* 2006;281:25994–26003. [PubMed: 16829680]
45. Berkovitch F, Nicolet Y, Wan JT, Jarrett JT, Drennan CL. Crystal structure of biotin synthase, an S-adenosylmethionine-dependent radical enzyme. *Science* 2004;303:76–79. [PubMed: 14704425]
46. Hanzelmann P, Schindelin H. Crystal structure of the S-adenosylmethionine-dependent enzyme MoaA and its implications for molybdenum cofactor deficiency in humans. *Proc Natl Acad Sci USA* 2004;101:12870–12875. [PubMed: 15317939]
47. Chen D, Tanem J, Frey PA. Basis for the equilibrium constant in the interconversion of L-lysine and L-β-lysine by lysine 2,3-aminomutase. *BBA – Proteins and Proteomics*. In press

Abbreviations

PLP	pyridoxal-5'-phosphate
SAM	S-adenosyl-L-methionine
EPSP	N-[2-hydroxyethyl-piperazine-N'-(3-propanesulfonic acid)]
dNTP	a 2'-deoxyribonucleotide-5'-triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EPR	electron paramagnetic resonance
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
PCR	

	polymerase chain reaction
PITC	phenylisothiocyanate
PMSF	phenylmethanesulfonyl fluoride
Tris	tris(hydroxymethyl)aminomethane
ENDOR	electron nuclear double resonance
ESEEM	electron spin echo envelope modulation
XAS	x-ray absorption spectroscopy
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

ALKAL	-----MNHDTTNSRQISIDRAKHLKLTIQDYLEIKDLIPKGLSRQVEIAKKQKILSHFGATEDNWNW	65
DESULF	-----MAIEFLPPNPRQASQARALELKQKVQSYNKRKETIPGGLALSEEFNENRDFLLDQLDADLEHWQDW	66
CARBOX	-----MNRARISQDEGLKQRELTRIGRSRLRERKVLGSGFEAWKILKQKEKILKLVGGTEEDWQDW	64
MOORE	-----MSIYPEKGRLMGR-EAKREIADRAAELKARIVDYLEEREKIASGLEAAAEIASEQRILLAYFGAGEAEWQDW	72
DREDUC	-----MSVHLKQEEFRLR-EKKRIALKRAELKARITDYLENKDQIKNGFEVQBYQYNRAQTLLNFNADNMQEWNW	72
PELOTO	MSCPKNKNGGVCMISIPVVEQRWRFSDEEKRAIALMAAELKDKISDYLEKKDLIKTGFDQFQETALNKRERLLKYPNSTESDWNW	85
SYNTRO	-----MLLRDELINIEIR-EMKREVSRLRADELKQEISDYLDIESTIETGMLRHLNHLNKEHILKFYEVSEENDWNW	72
THERMO	-----MSSTG-----SLTVEEKKIALQRAEELKKKIEPYLRASEKIEGTGFKLSEKFRENKEKIKNLPGATEEENWNW	68
CALDI	-----MEKLDVINNRE---RFEKLKKAIDYLEVKDTIKTGIDDEEKIEYQKRKILSYFGASEKDWENY	61
CDIFF	-----MN-----EQTRISLEAAELKSKIDDYIQAQKTINRGLEKEEBINKRKQKILSLNGTEEDWNW	60
CSUBT	-----MINRYELFKDVSD-----MINRYELFKDVSD-----ADWNW	20
ALKAL	QWQLNSRISDVTLTIIKLDDKEIEDIKVQGEFRWSVSPYYTTLIDDDN-KYCPILKMAIPHGYEI-ANTKGDTPMAEFTN	148
DESULF	KWQLKNRQDAENLSTLLPLTPKQRHEINEVGKAYRWAVSPYYLSLIDKDD-PQDPIRLQSLPSVEI-LDDSGEADPMGEYTS	149
CARBOX	RWQLKNRITTPVLRKILPLSDQVLEEVSKVYRFAISPYLLSLIDPDD-PDCGIKKQSIPILEV-LDDTGELDPMEAGTS	147
MOORE	RWQLTRITSVATLAEILPLTAEAEAILKVERTYRWAVSPYYLSLMGPE-PDCPIRRQALPSAAEL-EDNHGVLDPMEELTS	154
DREDUC	HWQMANRISKDVVISQLIDLSPAEEKAEIKVGRQYRWAVSPYYMALAMVSG-SGGPVWLQAIPCEIEV-KDRYGVDEPMGEYTS	155
PELOTO	RWQMSHRISDSGALAEPLGFNEMQCAQIKRVGMRYRWAVSPYYASLMGEDI-EHDPRLQVSPSIELE-NET-GHDPDMAEELTS	167
SYNTRO	AWQMRNRINDGNVLASILGLNEFEVQTIKRVSKVYRWAVSPYYLSLIDPENYAASPIYKQSVPSLHEI-IECKGEDPMGEEMSS	156
THERMO	RWQNRISDVTLEKIKVNLSEEEENIKRVSTRYRWAVSPYYASLMGPDN-PPCIRMRAIPSIKEL-TDKYGVDPMAEYTS	151
CALDI	KWQLKNRITSAKILKELLNLDEKAAQIEVAKIYRFAISPYLLSLIDPDD-PHCPKQKQVSPSSSEL-IEK-GELDPMEELTS	143
CDIFF	KWQLNSRITDVTLSKIIITLTKKEKEYIKVGTQFRWAVSPYYLSLIDPDD-ICDPIKLLSIPTHELE-EDQEQLDPMGEYTN	143
CSUBT	RWQVRNRITVTEELKKYIPLTKEEEGVAGQCVKSLRMAITPYLLSLIDPDD-PNDPVRKQAIPTALELNKAADLEDPHEEDTS	104
ALKAL	PAGSITRRYPDRLIINVINECAMYCRHCHRRRNIGETNDLHSTRVLEQESIDYIRDNPEIRDVLTITGGDALTLNSMLDWLLGELH	233
DESULF	PAPCITRRYPDRLIINVINICAMYCRHCHRRRNIGEIDLHETRANLEAALDYIRSNPEIRDVLTITGGDALLLSDQMLDWLLGELH	234
CARBOX	PVAATRRYPDRLIINVINICAMYCRHCHRRRNIGEVDRKTPREQIKKALYIREHKEIRDVLTITGGDALLLSDLELDWLLKELS	232
MOORE	PAPAITRRYPDRLIINVINICAMYCRHCHRRRNIGEVDRSRSRRELEQALQYIRQNEEIRDVLTITGGDALMLSDAMIDWLLTELD	239
DREDUC	PVEGVTRYPDRLIINVINICAMYCRHCHRRRNIGEIDVHKSRLVLEQALQYIRNKEIRDVLTITGGDALLLSDRQIEWLLTELD	240
PELOTO	PAPCVTRYPDRLIINVINICAMYCRHCHRRRNIGEVDRHSPPHESLVAALYIRKRNREIRDVLTITGGDALLLSDSKIDWLLSELD	252
SYNTRO	PAPRITRRYPDRLIINVINICAMYCRHCHRRRNIGETDNHAAHKLDEAALQYIKNNSEIRDVLTITGGDALMLSDRTLWLLGLELD	241
THERMO	PAPLITRRYPDRLIINVINICAMYCRHCHRRRNIGEVDPYPAKHEDIEAALYIRNNPEIRDVLTITGGDPLTLEDEKIDWLLSELD	236
CALDI	PTKIIITRRYPDRLIINVINICAMYCRHCHRRRNIGETDTHASLDDITDAEYVAQNPNIRDVLTITGGDALMLSDIELEWILRSR	228
CDIFF	PAGCITRRYPDRLIINVINECAMYCRHCHRRRNIGQDSDHKSIAIQESIDYIRENEEIRDVLTITGGDALTLKDDYLEWILRSR	228
CSUBT	PVPGLTHRYPDRVLLITDMSYCRHCHRRRPAQSDSDMPMERIDKAIDYIRNTPQVIRDVLTITGGDALVSDTELEYIARLR	189
ALKAL	AIPSDVYIRLGSRTLVTMPQRITDKLINILKKYPPIFINTQFNHPMEITEESKACDRLSNAGIPLGNQAVLLNGINNKNFVMR	318
DESULF	EIKHVEIKRIGTRVPVTLPMRITDELCAILEKYPPLIYINTQFNHPQEVTEETKKAADRLIKAGVILGNQAVLLKGINNQPEIMKR	319
CARBOX	EIPHVEIKRIGTRVPVTLPMRITDNLVKILKKYPPIYINTQFNHPREVTPAEKAVDKLIEAGVVLGNQAVLLKGVNDNPVIMEK	317
MOORE	NI PHVEIKRIGTRVPVTLPMRITPELCLVLAHPPIYINTQFNHPREVTAARKEACDRLVQAGVVLGNQAVLLKGVNNHPFVMRK	324
DREDUC	NI PHVEIKRIGTRVPVTLPMRITPELCKLENHPPIYINTQFNHPLEVTPAEKACDRLVQAGVVLGNQAVLLKGINNQPDVVMRK	325
PELOTO	KIKHVEIKRIGTRVITLPMRITPELCLVLAHPPIYINTQFNHPREVTPESKQACDRLVQAGVVLGNQAVLLKGVNDNPVIMKK	337
SYNTRO	AISHVEIKRIGTRVPVTLPMRITANLCAVLKRHTPIYINTQFNHPLEVTPAEKQACDRLVQAGVVLGNQAVLLKGINNDNVHVMKK	326
THERMO	KIPHVEIKRIGTAAPVTFPQRITDELCKILTKHLPYIYINTQFNHPKEVTEAEKACFCLARAGVALGNQAVLLKGINNDNVHVMKK	321
CALDI	QIPHVEIKRIGTRAPVTLPMRITKELVDMKKYHPYIYINTQFNHPREITKESKACCEMLADGGIPLGNQAVLLNGVNNKDYVVR	313
CDIFF	EIPHDVYIRLGSRTLVTMPQRITDEFENMLKKYHPVYINTQFNHPMEITEESKACEKLANAGVPLGNQAVLLNGVNNKDYVVR	313
CSUBT	EIPHVEIVRIGSRTLVTMPQRITPELVNMLKKYHPVWLNTQFNHPNGITEESTRACQLLADAGVPLGNQAVLLRGVNDNVHVMKE	274
ALKAL	LNHELLKCRVPPYIFHAKVKGTHFVITGSHFNTSVDGIEIMEYLRGYTSGMAIPTIYNAPGGGKTIPILPQYILSRGSHSIKIRTWD	403
DESULF	LNQELLKIRVPPYIFHAKVKGTHFIPRIQDGLRIMENLRGYTSGLAIPYIYNAPGGGKTIPILPQYILSLNDEAVIRITWE	404
CARBOX	LNHELLKIRVPPYIFHAKVKGTHFVPKIEDGLRIMESLRGYTSGLAIPYIYNAPGGGKTIPILPQYIELSEEAVALRNWE	402
MOORE	LNQELLKIRVPPYIFHAKVKGTHFISIEEGVEIMDKLRGYTSGLAIPYIYNAPGLGKTIPILPQYIARNDHQVILRTWE	409
DREDUC	LNQSLTIRVPPYIFHAKVKGTHFITGVDGDIAMEQLRGYTSGLAIPYIYNAPNGYKTPVLPQYIENKNDQVILRTWE	410
PELOTO	LNQELLKIMVPPYIFHAKVKGTHFITAVDEGISIMEQLRGYTSGLAIPYIYNAPNGYKTPVLPQYILGNNGDKILRTWE	422
SYNTRO	LNQELLKIRVPPYIFHAKVKGTHFISPVTGLDIMKHLRGYTSGLAIPYIYNAPGGYKTPVNPVYVLLINENVEIISTWQ	411
THERMO	LNHELLRIMVPPYIFHAKVKGTHFVITVQDGLIMEQLRGYTSGLAIPYIYNAPGHGKTIPVLPQYILLMVGKEYVILIRNWE	406
CALDI	LNQQLKIRVPPYIFHAKVKGTHFVVTIEBGMIEIESLRGYTSGMAIPTIYNAPGGGKTIPIMPNYLLYFGKGKVVFRNWE	398
CDIFF	LNQELLKIRVPPYIFHAKVKGTHFNTSVDGIEIMEYLRGYTSGMAIPTIYNAPGGGKTIPILPQYLVSKGTDVYMLRTWE	398
CSUBT	LVNKLKIRVPPYIYQQLSLGLEHFRTPFVSKGIEIEGLRGHTSGYCVPTFVYNAPGGGKTIPVMPNYIISQSHDVILRNFE	359
ALKAL	GEVIDYPNHPTPIEETLK-----	422
DESULF	GKVVHYPNH-----	413
CARBOX	GRIIRYPN-----	410
MOORE	KRIIFYSNLGRQKEQA-----	425
DREDUC	KRIIPYNSGKH-----	422
PELOTO	NRVLEYSFLPGGE-----	435
SYNTRO	GKTFNYPHNN-----	422
THERMO	GKVFEPYNGFPDD-----	419
CALDI	GEVFEVENG-----	407
CDIFF	GKVIKMEDEPAVDIKKLIKQAQD-----	422
CSUBT	GVITTSSEPNITPGCNCVCTGKKKVHKGVAGLLNGBGMALEPVGLEKRNKRHVCE	416

Figure 1. Aligned amino acid sequences for putative aminomutases

Amino acid sequences of proteins from the following bacterial sources were obtained by a search of the microbial genomic database of translated proteins with the amino acid sequence of *C. difficile* glutamate 2,3-aminomutase. Amino acid sequences were aligned with the ClustalW program (34). 1. ALKAL – *Alkaliphilus metalliredigenes* QYMF (NZ AAKU01000055), 2. DESULF – *Desulfotobacterium hafniense* Y51 (NC 007907), 3. CARBOX – *Carboxydotherrus hydrogeniformans* Z-2901 (NC 007503), 4. DREDUC – *Desulfotomaculum reducens* MI-1 (ZP01148336), 5. PELOTO – *Pelotomaculum thermopropionicum* SI (BAAC01000015), 6. SYNTRO – *Syntrophomonas wolfei* str. Goettingen (AAJG01000011.1), 7. THERMO – *Thermoanaerobacter tengcongensis* MB4 (NP

622830), 8. CALDI – *Caldicellulosiruptor saccharolyticus* DSM 8903 (NZ AALW01000017), 9. CDIFF – *Clostridium difficile* (ZP 01230643), 10. CSUBT – *Clostridium subterminale* SB4 (Q9XBQ8).

Figure 2. SDS Polyacrylamide gel electrophoresis of recombinant *C. difficile* glutamate 2,3-aminomutase produced in *E. coli*

Lane 1: Protein standards (Benchmark Protein Ladder, Invitrogen) 1 g each protein. Lane 2: *E. coli* cell extract 30 g. Lane 3: Purified recombinant *C. difficile* glutamate 2,3-aminomutase 4 g. Arrow indicates the marker for a 50,000 MW protein.

Figure 3. Interconversion of L-glutamate and β -glutamate by *C. difficile* glutamate 2,3-aminomutase

Separation and identification by HPLC. Assay conditions as described in Methods. A.) L-glutamate as substrate. Initial conditions: L-glutamate – 5 mM, enzyme 3.7 M (subunit). B. β -glutamate as substrate. Initial conditions: β -glutamate – 5 mM, enzyme 3.7 M (subunit).

Figure 4. UV-Visible spectrophotometry of *C. difficile* Glutamate 2,3-aminomutase
Purified enzyme as isolated (0.45 mg/mL) in 0.02M sodium EPPS pH 8.0 buffer was evaluated anaerobically with a Hewlett-Packard Diode Array Spectrophotometer (Model 8452A).

Figure 5. EPR spectroscopy of *C. difficile* glutamate 2,3-aminomutase

See Methods section for description of sample preparation. A) L-glutamate 50 mM, B) β -glutamate 50 mM, C) (2- ^{13}C)-DL-glutamate 100 mM; D) (2,3,3,4,4- d_5)-L-glutamate 50 mM, E) L-glutamate 50 mM *S*-(5'-adenosyl)-L-methionine omitted, F) L-glutamate 50 mM, sodium dithionite omitted.

Figure 6. The likely chemical mechanism for the action of glutamate 2,3-aminomutase

The catalytic cycle begins with the internal enzyme (E) aldimine of PLP and the active site lysine, illustrated at left (center). Transaldimination with L-glutamate generates the external aldimine of PLP with L-glutamate, freeing the active site lysine. The nearby S-adenosylmethionine is reversibly cleaved to the 5'-deoxyadenosyl radical by reaction with the [4Fe-4S]¹⁺ cluster, and the radical abstracts a hydrogen atom from C3 of the glutamate side chain, forming radical 1 and 5'-deoxyadenosine, which remains bound to the active site. Radical 1 undergoes isomerization to radical 2 and thence to radical 3, the β-glutamate-related radical, which abstracts a hydrogen atom from the methyl group of 5'-deoxyadenosine. The resulting external aldimine of PLP and β-glutamate undergoes transaldimination with the active site lysine to release β-glutamate and regenerate the internal PLP aldimine for another catalytic cycle.

Lysine 2,3-aminomutase

Glutamate 2,3-aminomutase

Figure 7. Proposed hydrogen bonded contacts of glutamate and glutamate 2,3-aminomutase

Based on the hydrogen bonded contacts of lysine 2,3-aminomutase with lysine shown at the left (4), and the aligned amino acid sequences of lysine and glutamate 2,3-aminomutases in Fig. 1, the complementary hydrogen bonded contacts of glutamate and the 2,3-aminomutase shown on the right can be deduced.

Table 1

Properties of glutamate 2,3-aminomutase

Parameter	Value
K_m (mM) ^a	2.3 ± 0.2
k_{cat} (s ⁻¹) ^a	6.1 ± 0.2
k_{cat}/K_m (s ⁻¹ M ⁻¹) ^a	2.7×10^3
$K_{eq} = [\beta\text{-Glu}]/[\text{L-Glu}]$ ^b	15.7 ± 0.5
Fe content (subunit ⁻¹)	2.9 ± 0.2
S ²⁻ content (subunit ⁻¹)	2.5 ± 0.2
PLP content (subunit ⁻¹)	1.1 ± 0.1
ϵ_{280} (M ⁻¹ cm ⁻¹)	8.7×10^4
ϵ_{420} (M ⁻¹ cm ⁻¹)	1.7×10^4

^a pH 8.0, 37 °C^b 24 °C; 24 determinations, 12 in each direction