Cloning, Sequencing, Heterologous Expression, Purification, and Characterization of Adenosylcobalamin-dependent D-Lysine 5,6-Aminomutase from Clostridium sticklandii*

(Received for publication, August 31, 1999, and in revised form, October 12, 1999)

Christopher H. Chang‡ and Perry A. Frey§

From the Institute for Enzyme Research, Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53705

D-Lysine 5,6-aminomutase from Clostridium sticklandii catalyzes the 1,2-shift of the ϵ-amino group of d-lysine and reverse migration of C5(H). The two genes encoding 5,6-aminomutase have been cloned, sequenced, and expressed in Escherichia coli. They are adjacent on the Clostridial chromosome and encode polypeptides of 57.3 and 29.2 kilodaltons. The predicted amino acid sequence includes a conserved base-off 5'-deoxyadenosylcobalamin binding motif and a 3-cysteine cluster in the small subunit, as well as a P-loop sequence in the large subunit. Activity of the recombinant enzyme exceeds that of the 5,6-aminomutase purified from C. sticklandii by 6-fold, presumably due to the absence of bound, inactive corrinoids in the recombinant enzyme. The Km values for adenosylcobalamin and pyridoxal 5'-phosphate are 6.6 and 1.0 μM, respectively. ATP does not have a regulatory effect on the recombinant protein. The rapid turnover associated inactivation reported for the enzyme purified from Clostridium is also seen with the recombinant form. Aminomutase activity does not depend on structural or catalytic metal ions. Electron paramagnetic resonance experiments with [15N-dimethylbenzimidazole]adenosylcobalamin demonstrate base-off binding, consistent with other B12-dependent enzymes that break unactivated C—H bonds.

Clostridium sticklandii ferments lysine to acetic acid, butyric acid, and ammonia. There are two separate pathways of lysine catabolism in this bacterium, which differ for L- and D-lysine (1). The first committed step in D-lysine catabolism is catalyzed by D-lysine 5,6-aminomutase. The enzyme can be purified as a 170-kDa complex of 55- and 30-kDa subunits. The predicted amino acid sequence includes a conserved base-off 5'-deoxyadenosylcobalamin binding motif and a 3-cysteine cluster in the small subunit, as well as a P-loop sequence in the large subunit. Activity of the recombinant enzyme exceeds that of the 5,6-aminomutase purified from C. sticklandii by 6-fold, presumably due to the absence of bound, inactive corrinoids in the recombinant enzyme. The Km values for adenosylcobalamin and pyridoxal 5'-phosphate are 6.6 and 1.0 μM, respectively. ATP does not have a regulatory effect on the recombinant protein. The rapid turnover associated inactivation reported for the enzyme purified from Clostridium is also seen with the recombinant form. Aminomutase activity does not depend on structural or catalytic metal ions. Electron paramagnetic resonance experiments with [15N-dimethylbenzimidazole]adenosylcobalamin demonstrate base-off binding, consistent with other B12-dependent enzymes that break unactivated C—H bonds.

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* This work was supported in part by NIDDK National Institutes of Health Grant DK28607. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) and translated open reading frames reported in this paper have been submitted to GenBank™/EMBL Data Bank with accession number AF104259.

‡ Supported by a National Science Foundation predoctoral fellowship.

§ To whom correspondence should be addressed. Tel.: 608-262-0055; Fax: 608-265-2904; E-mail: frey@enzyme.wisc.edu.

EXPERIMENTAL PROCEDURES

Chemicals—Tryptone and yeast extract were from Difco for Clostridium medium or Acumedia (Baltimore) and Marcor Development Corp. (Hannong, NJ), respectively, for E. coli medium. Genomic DNA was isolated with a Genomic Tip from Qiagen, and plasmid DNA was prepared for automated fluorescent sequencing with the Qiagen mini prep kit. Competent JM109 cells (>10⁸ colony forming units/μg), plasmid Maxiprep kits, all plasmid vectors, marker ladders, and most restriction enzymes were from Promega Corp. (Madison, WI). HindIII was from Amersham Pharmacia Biotech, SpeI was from Amersham Pharmacia Biotech or New England Biolabs, and NdeI was purchased from Roche Molecular Biochemicals. Cloned Pyrococcus furiosus DNA polymerase was from Stratagene. Competent DH5αMCR and BL21(DE3) E. coli cells were from Life Technologies, Inc. GeneScreen cationic nylon membranes were from NEN Life Science Products. Polyvinylidifluorid membranes were from Bio-Rad. Amido Black stain was from Sigma. All chemicals were of molecular biology grade or higher.

Clostridium Growth Conditions—C. sticklandii was obtained as freeze-dried cells from the American Type Culture Collection (ATCC, Manassas, VA). After reconstitution, cells were grown anaerobically in medium consisting of, per liter: 4.5 g tryptone, 4.5 g of yeast extract, 1.4 g of KH₂PO₄, 1.0 g of l-lysine-HCl, 25 mg of CoCl₂•H₂O, 10 mg of

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CaCl₂·2H₂O, 0.20 g of MgSO₄·7H₂O, 0.9 g of KOH, and either 0.5 g of sodium thiglycolate + 5 mM sodium dithiole (cultures < 60 liters) or 20 mg of sodium dithionite (60 liter culture). A trace of methylene blue was included as a redox indicator. Cells were propagated in 100 ml of anaerobic bottle cultures, and a fresh 100-ml culture used to inoculate a 3-liter starter culture. The overnight 3-liter culture was used to inoculate anaerobic bottle cultures, and a fresh 100-ml culture used to inoculate a 60-liter fermentor at the University of Wisconsin, Department of Biochemistry Pilot Plant. The culture was grown to an A₅₆₀ of 0.4, harvested through a Sharples continuous-flow centrifuge, and frozen in liquid nitrogen.

**Purification of Clostridial D-Lysine 5,6-Aminomutase**—Protein concentrations were determined by the method of Lowry et al. (6). Serum albumin was used as a standard. Denaturing polyacrylamide gels were stained with Coomassie Blue. Clostridial 5,6-aminomutase was purified by a modification of the method of Baker et al. (5). All manipulations were under subdued light, and vessels containing protein were covered with aluminum foil or black plastic to protect against AdoCbl photolysis. One hundred grams of frozen C. sticklandii were thawed in 20 mM KPO₄, 2 mM NaEDTA, pH 7.2, to which 100 μL of 0.5 mM phenylmethylsulfonyl fluoride/acetone was added. After centrifuging 20 min at 6,000 × g at 4 °C, the rinsed pellet was suspended in 20 mM KPO₄, pH 7.2, 1 mM dithiothreitol, 1 mM MgCl₂, then 50 μL of 0.5 mM phenylmethylsulfonyl fluoride/acetone, 10 mg of lysozyme, and several crystals of deoxyribonuclease I (Sigma) were added. The cells were incubated in the same conditions for 1 h, then 1 mL of enzyme in the suspension sonicated once for 1 min at high power with a Heat Systems Ultrasonic, Inc. Sonicator. After centrifugation, the supernatant fluid was dialyzed twice for 3–4 h against 1 liter of 20 mM KPO₄, pH 7.2, 1 mM dithiothreitol, 2 mM NaEDTA, hereafter referred to as standard buffer. The dialyzed solution was loaded onto a 5 × 30-cm DE52 column equilibrated in standard buffer and attached to a Amersham Pharmacia Biotech PPLC system. The column was washed with 600 mL of standard buffer, 0.1 mM NaCl and 600 mL of standard buffer, 0.2 mM NaCl, and the enzyme was eluted with a 1.2-liter linear (0.3–0.5 M NaCl) gradient into 10-ml fractions. Fractions that were visibly pink or orange during brief light exposure were assayed as described (3) with minor modifications (see below) and analyzed by SDS-PAGE. Pooled fractions were concentrated to 500 μL by ultrafiltration, loaded onto a Hi-Prep 26/60 Sephacyr S-200 HR column (Amersham Pharmacia Biotech), and eluted with standard buffer, 0.1 mM NaCl into 2-ml fractions. Fractions pooled based on activity and gel purity were loaded onto a Mono Q HR 16/10 column (Amersham Pharmacia Biotech) equilibrated in standard buffer. A 0–1 M NaCl gradient was run at 5 mL/min, and fractions pooled based on activity and gel purity were loaded onto a 2-ml fractions. Two bands therefrom were chosen for N-terminal analysis. Sequencing, and Synthesis Facility for N-terminal sequencing. The pCC-4 insert was gel-purified and used as a template for probe synthesis with [32P]dATP (13). HindIII-digested Clostridial genomic DNA was Southern hybridized against this probe, and an approximate 5-kbp band was detected by exposure to Kodak Bio-Max film. DNA in

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>31/C</td>
<td>ATGGAARAAARGARTT</td>
<td>1915–1931</td>
</tr>
<tr>
<td>31/AC</td>
<td>AAYCTGTTTTCCTCA</td>
<td>1915–1931</td>
</tr>
<tr>
<td>51/C</td>
<td>ATGAGGAAVYTTYAAYTTGYATTAA</td>
<td>319–347</td>
</tr>
<tr>
<td>51/AC</td>
<td>TTTTARTC1RITTAAYTCTYTCCAT</td>
<td>319–347</td>
</tr>
<tr>
<td>31/kD3/AC</td>
<td>TYYTDTAARCTYCRYT</td>
<td>2398–2417</td>
</tr>
<tr>
<td>NdeI-Primer</td>
<td>GGGGATCCATAGAAGGAAG</td>
<td>307–326</td>
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<tr>
<td>BamH-Primer</td>
<td>AGACGATCTTACACGAT</td>
<td>1331–1350</td>
</tr>
<tr>
<td>Bam3PCR</td>
<td>CAAGATATATGCGATCCAAATTAG</td>
<td>2655–2711</td>
</tr>
</tbody>
</table>

this size range was excised from another gel and ligated into HindIII-digested pGEM-3Zf(−). The resulting construct (pCC-9) was transformed into DH5α MCR cells. In situ hybridization on GeneScreen membranes with 32P-labeled probe led to three independent isolate strains: CC-8, CC-10, and CC-11.

**DNA Sequencing—** Thermocycle double-stranded DNA sequencing was performed using materials and protocols from the University of Wisconsin Biotechnology Center. Reactions were run through a Amersham Pharmacia Biotech G-50 spin column, dried, and submitted for analysis. Some convenient deletions next to the T7 universal primer site were made by restriction/religation, and coding strand sequence was determined by sequencing the deleted plasmids from the T7 site or primer walking with pCC-9 as template. DNA sequences were verified by sequencing the anticoding strand of pCC-10 and pCC-11. Sequence information was manipulated with the Wisconsin Package, version 7 (14).

**Expression Construct**—The coding regions for the subunits of lysine 5,6-aminomutase, defined as the isolated open reading frames beginning with the experimentally determined N-terminal amino acid sequences, were PCR amplified from pCC-9 in two pieces using high fidelity TaKaRa polymerase NdeI-Primer introduced an NdeI site at the first codon of the large subunit and simultaneously changed the native GTG initiation codon to ATG. The reverse PCR primer BamH-Primer was designed around an internal BamHI site in the Clostridial DNA. The resulting amplified DNA was cloned into the Smal site of pGEM-3Zf(−) to produce pCC-20. The second half of the coding DNA was amplified between coding primer Bam2PCR and mutagenic anticoding primer Bam3PCR that introduced a BamHI site near the 3' end of the small subunit coding DNA. This PCR product was also cloned into pGEM-3Zf(−) as before to produce pCC-24. The expression construct was made by ligating the NdeI-BamHI fragment of pCC-20 into the NdeI-BamHI site of pET-9a, isolating the resulting construct in DH5α MCR, then cloning the BamHI insert from pET-9a into the BamHI site of pET28a (Novagen). The NdeI-Primer introduced a Ndel site near the 3' end of the small subunit. The entire insert was sequenced to check for PCR-induced mutations, then the complete plasmid was transformed into BL21(DE3) to produce strain CC-28. A control strain CC-29 was made by transforming BL21(DE3) with pET-9a.

To test induction properties, 2 ml of LB + 30 μg/ml kanamycin was inoculated with either CC-25 or CC-29 and grown 12 h. Each culture was induced with 12 μg of n-lactose and grown for another 2 h. Samples were taken at zero, 30, 60, and 120 min after induction for gel analysis (Fig. 1).

Assays of recombinant enzyme samples initially consisted of 100 mM NaEPPS, pH 8.5, 5 mM MgCl₂, 40 mM NH₄Cl, 50 mM β-mercaptoethanol, 40 μM PLP, 20 μM adenosylcobalamin, and 20 mM D-l-lysine/HCl in a final volume of 50 μL. This composition differed from that reported by Morley and Stadtman (3) in the replacement of Tris by EPPS buffer to eliminate the potential reactivity of neutral Tris. In addition, [Mg++] was made equal to [ATP], and 8 mM dithiothreitol was replaced by 50 mM β-mercaptoethanol. As characterization developed, conditions were modified to maintain saturation with essential components while eliminating non-essential standard components. The reaction was run with 100 mM NH₄EPPS, pH 8.5, 5 mM β-ME, 40 μM PLP, and 100 μM AdoCbl. Acid-quinoned assays were analyzed by thin-layer chromatography of radiolabeled substrates and products (3).

**Expression and Purification of the Recombinant Enzyme**—A 1-liter culture of CC-28 was produced by gentle shaking (~200 rpm in an un baffled, 1-liter Erlenmeyer flask) for 16 h at 37 °C. Cells were harvested by centrifugation for 20 min at 5,000 × g, 4 °C, and lysed by

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Fig. 1. 12% SDS-PAGE of whole CC-28 and CC-29 cells. Each set of 5 lanes represents 0, 30, 60, 90, and 120 min after induction of 12-h liquid nitrogen.

Table II

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><strong>pCC-4</strong></td>
<td>pCC-4pGEM-T/500-bp PCR product from C. sticklandii genomic DNA</td>
</tr>
<tr>
<td><strong>pCC-9</strong></td>
<td>pGEM-3Zf–v5-kbp probe-positive HindIII fragment from C. sticklandii genomic DNA</td>
</tr>
<tr>
<td><strong>pCC-20</strong></td>
<td>PCR-amplified upstream portion of kamDE between BamHI mutagenic site and pre-existing internal BamHI site, cloned into Smal site of pGEM-3Zf(–)</td>
</tr>
<tr>
<td><strong>pCC-24</strong></td>
<td>PCR-amplified downstream portion of kamDE between internal BamHI site and extreme 3′ mutagenic BamHI site, cloned into Smal site of pGEM-3Zf(–)</td>
</tr>
<tr>
<td><strong>pCC-28</strong></td>
<td>pET-9a containing PCR fragment 3 (pCC-20 insert) and PCR fragment 4 (pCC-24 insert) in proper 5′–3′ orientation for KamDE expression</td>
</tr>
<tr>
<td><strong>CC-9</strong></td>
<td>DH5αmCER cells containing pCC-9</td>
</tr>
<tr>
<td><strong>CC-10</strong></td>
<td>Independent isolate of CC-9</td>
</tr>
<tr>
<td><strong>CC-11</strong></td>
<td>Independent isolate of CC-9</td>
</tr>
<tr>
<td><strong>CC-28</strong></td>
<td>pCC-28 in BL21(DE3) cells</td>
</tr>
<tr>
<td><strong>CC-29</strong></td>
<td>pET-9a in BL21(DE3) cells</td>
</tr>
</tbody>
</table>

![Image](http://www.jbc.org)
Asp relative to Arg, Lys, and His, which rationalizes the acidic character of the enzyme as observed on ion exchange columns at pH 7.5.

Comparison of the 5,6-aminomutase subunit amino acid sequences to GenBank™ entries reveals a region in KamE with similarity to B12-dependent mutases, including Clostridial glutamate mutase, Archaeoglobus methylmalonyl-CoA mutase, and 2-methyleneglutarate mutase from Clostridium barkerii (Fig. 3). Notably, the conserved histidine of these "base-off" mutases that replaces the 5,6-dimethylbenzimidazole bottom axial ligand of adenosylcobalamin upon binding is present in the conserved GXXDXHXXG motif. This putative adenosylcobalamin binding sequence correlates with previous observations that the small subunit of the aminomutase binds corrinoids (6).

The large subunit possesses a distinctive group of three cysteine residues in the motif Cys-X2-Cys-X2-Cys (C234a, C237a, C241a). This sequence may be compared with the Cys-X2-Cys-X2-Cys found ligating a [4Fe-4S] cluster in the corrinoid/iron-sulfur protein (16) and the structural zinc binding motif Cys-X2-Cys-X2-Cys in alcohol dehydrogenase (17). However, the spacing between the second and third cysteines in KamD differs from both. Unlike L-lysine 2,3-aminomutase, KamDE does not contain iron or inorganic sulfide, nor does it display spectral characteristics of iron-sulfur centers. The significance of these grouped thiols, if any, is unknown.

Expression and Purification of the Recombinant Enzyme—As seen in Fig. 1, CC-28 continuously expresses the 5,6-aminomutase polypeptides from the pET vector construct during vigorous aerobic growth at 37 °C. These peptides were found entirely as inclusion bodies. Under less vigorous agitation, however, a significant portion of these peptides are present as soluble, active enzyme in a cell-free extract (Fig. 4). A liter of LBK culture usually provides $50 \text{mg}$ of purified, soluble apo-B12 KamDE with a specific activity greater than that seen with enzyme purified from Clostridium (Table III).

ATP Dependence—Allosteric regulation by ATP was seen in early studies of the 5,6-aminomutase (3). However, as shown in Fig. 5, 5 mM ATP does not significantly affect lysine saturation properties of the recombinant KamDE. Any effects seen at higher concentrations would have questionable mechanistic significance in vivo. Unlike what was seen for the combined E1-E2 system, no change from sigmoidal to hyperbolic kinetics occurs for the purified recombinant enzyme. The minor enhancement of $V_{\text{max}}$ with added MgATP in Fig. 5 was not ob-
served consistently in subsequent studies, and is not considered significant.

**Metal Content**—The purified enzyme contains a substantial quantity of metal ions, primarily \( \text{Zn}^{2+} \). When dialyzed against a mixture of chelators, however, the metal content was lowered to \( \approx 4\% \) of tetramer present. The activities and thermal stabilities of untreated and metal-depleted KamDE were compared using metal-free reagents (excepting the \( \text{Co}^{2+} \) in AdoCbl). As shown in Fig. 6, almost no activity difference was seen between the two preparations with \( 37 \, ^\circ \mathrm{C} \) preincubation, minimizing the possibility of an essential catalytic metal. Chelation also does not result in greater temperature sensitivity, as might be observed upon removal of a structural metal; in fact, the chelated enzyme shows slightly greater temperature stability. While not definitive evidence against the presence of a structural metal in KamDE in vivo, the similarity in thermal lability between as purified and metal-depleted KamDE does not support such presence.

**Inactivation during Catalysis**—The 5,6-aminomutase purified from *C. sticklandii* was found to undergo rapid inactivation in the absence of the activating enzyme \( E_2 + \text{ATP} \) (6). To examine inactivation of the recombinant enzyme, KamDE was incubated with and without 20 mM substrate for various times before assaying with saturating lysine. Fig. 7 shows an exponential drop in measured activity when substrate is present, with a decay coefficient of 0.31 min \(^{-1}\). The curve fitted to the data in Fig. 7 includes a constant phase to account for apparent residual activity at long times. When \( \text{D-lysine} \) is absent, activity is stable in the presence of AdoCbl, implying that the inactivation process is linked to \( \text{D-lysine} \) turnover. Without AdoCbl but with \( \text{D-lysine} \) present, the enzyme loses activity more slowly over time at \( 37 \, ^\circ \mathrm{C} \) after an approximately 2-min lag period. This loss is consistent with the data in Fig. 6, where KamDE is seen to be significantly thermally inactivated within 5 min at \( 37 \, ^\circ \mathrm{C} \) in the absence of added cofactors. We attribute the thermal instability to the absence of the potentially major structural determinant AdoCbl. Nevertheless, the enzyme maintains activity at \( 37 \, ^\circ \mathrm{C} \) when AdoCbl is present, and the rate of activity loss is insubstantial compared with the catalytic inactivation. The inactivation occurs regardless of the presence of oxygen, suggesting that a process besides oxygen scavenging of catalytic radicals is responsible. The detailed mechanism of this inactivation is under investigation.

**AdoCbl Binding Mode**—Fig. 8 shows the EPR spectra of photolyzed AdoCbl, photolyzed [\( ^{15}\text{N}-\text{DMB} \)]AdoCbl in which the dimethylbenzimidazole moiety is approximately 60% enriched...
with $^{15}$N, and KamDE reconstituted with $[^{15}$N-DMB]AdoCbl and treated with methylhydrazine. The features of interest are the sawtooth-like superhyperfine splittings overlying the hyperfine splittings from the cobalt I $^{57/2}$ nucleus. The superhyperfine coupling is between the electron spin on the corrinoid cobalt atom and its lower axial ligand, and so serves as a probe into the cobalt ligation environment. When $^{14}$N is the lower axial ligand to cob(II)alamin, these superhyperfines are 1:1:1 triplets due to the I $^{1/2}$ spin of the nitrogen nucleus (Fig. 8A).

If this nitrogen nucleus is replaced by $^{15}$N, the superhyperfine features become doublets. In cob(II)alamin, these doublets fortuitously overlap the outer triplet features of $^{14}$N because the $^{15}$N splitting is twice that of $^{14}$N, as seen in Fig. 8B.

When apo-B$_{12}$ KamDE is reconstituted with $[^{15}$N-DMB]AdoCbl, the resulting cob(II)alamin spectrum demonstrates only triplet superhyperfines (Fig. 8C). This can only arise if the lower $^{15}$N-enriched DMB ligand has been replaced by another nitrogen nucleus. In all other cases of so-called base-off B$_{12}$ enzymes, where the DMB base has been replaced during binding, the replacing ligand has been hypothesized or demonstrated to be the His of a conserved GXGXHXXG sequence. The presence of this sequence in KamDE combined with the observation of triplet superhyperfines with $[^{15}$N-DMB]AdoCbl leads us to propose DMB base-off AdoCbl binding for the 5,6-aminomutase.

**DISCUSSION**

We have cloned, sequenced, and expressed the C. sticklandii genes encoding d-lysine 5,6-aminomutase and have addressed several questions regarding the enzyme’s properties. Aminomutase purified from Clostridium could not be resolved of bound corrinoids, so production of the recombinant form in an organism that does not produce intracellular corrinoids was essential in studying AdoCbl dependence, maximum specific activity, turnover based inactivation, and AdoCbl binding mode as discussed below.

Several lines of evidence convince us that we have cloned and expressed the correct constituent polypeptides of D-lysine 5,6-aminomutase. The expressed and purified polypeptides catalyze the D-lysine 5,6-aminomutase reaction. Identically with the enzyme from C. sticklandii, the recombinant enzyme displays two bands on SDS-PAGE gels. We have not observed an additional small subunit that has been reported before (6); although KamDE is sufficient for aminomutase catalysis, an additional subunit could serve a regulatory function. By virtue of the fact that the N-terminal sequences determined from these two SDS-PAGE bands were found in adjacent open reading frames within a single operon, there is little question of incidental co-purification of a contaminating protein from Clostridium or that these are true subunits of a single enzyme complex. Self-consistent DNA sequences from both DNA...
strands from three independent isolates convinces us that the nucleotide and predicted amino acid sequences are correct; furthermore, the apparent molecular masses from sequence information are very similar to the estimated $M_r$ values from SDS-PAGE.

Three interesting sequence features are present in the KaMDE subunits: a consensus P-loop sequence (AVLNTGKT, 83–90), a three-cysteine motif (CNYCSGLC, 234–241), and a conserved base-off $B_{12}$ binding motif (GTDAHTVG, 129–136). If the P-loop is involved in binding the phosphate present in the AdoCbl side chain containing the DMB ligand, the extremely tight $B_{12}$ binding may have a partial explanation. The consensus base-off sequence is in the $b$ subunit, so one may be reasonably certain that the cobalt of the corrinoid is on KamE. If the lower ligand arm of $B_{12}$ is buried during binding, and the phosphate binding portion is part of KamD, then AdoCbl may serve to bridge the subunits of KamDE. If this were the case, loss of $B_{12}$ might facilitate subunit dissociation.

Our expression is based on an inducible T7 system (18). In our hands, expression appears constitutive and to very high levels, a reality that has several advantages. The cultures do not need to be induced, so production of the enzyme is simple and very reproducible. Expression is at a low, constant level rather than a large spike, so demand is moderate for charged tRNA and other components necessary for transcription and translation. This gentle demand may be one reason that the codon usage differences between $C. sticklandii$ and $E. coli$ do not prevent expression in this case, the cells can keep up with tRNA charging demands, whereas a spike of expression would quickly deplete rare charged tRNAs and lead to ribosomal pausing and incomplete translation. The different distribution of the AGA arginine codon in the D-lysine 5,6-aminomutase genes relative to the L-lysine 2,3-aminomutase gene (19), rare in $E. coli$, may also bear on expression. 3

A key feature of our expression system is slow growth of $E. coli$ with submaximal culture agitation. When CC-28 is grown using baffled 2-liter Erlenmeyer flasks designed to increase culture oxygenation, almost all of the 5,6-aminomutase is present as insoluble inclusion bodies at 37 °C. Slower cell division might allow an otherwise limiting component in the protein translation system, e.g., a chaperone, to keep up with 5,6-aminomutase expression. Interestingly, apo-$B_{12}$ KamDE exhibits

3 AGA codons are clustered in the kamA gene specifying Clostridial L-lysine 2,3-aminomutase (19). This gene is poorly expressed in $E. coli$, and its expression is toxic to the cells. Expression is dramatically enhanced and toxicity relieved by co-expression of argU, which encodes the appropriate rare arginine tRNA in $E. coli$.  

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**FIG. 3.** Sequence comparison between d-lysine 5,6-aminomutase $\beta$-subunit and a variety of $B_{12}$Z-dependent enzymes and one uncharacterized open reading frame. Identical residues are shown as white on black, and conservative replacements are black on gray. Sequence identification numbers are given in parentheses for the NCBI Entrez protein database. 1, C. barkerii $B_{12}$-dependent 2-methyleneglutarate mutase (543481); 2, C. tetanomorphum glutamate mutase MutS (49240); 3, Clostridium cochlearium methylaspartate mutase component S (2127335); 4, Archaeoglobus fulgidus methylmalonyl-CoA mutase a-subunit C terminus (2848365); 5, E. coli $B_{12}$-dependent homocysteine $N_2$-methyltetrahydrofolate transmethylese (409794); 6, unknown $E. coli$ 91-amino acid open reading frame (1786523).

**FIG. 4.** Purification of d-lysine 5,6-aminomutase. Lanes from left to right represent the successive purification stages outlined in Table III.

**TABLE III**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (IU)</th>
<th>Specific activity (IU/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
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<td>458</td>
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<td>2.05</td>
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<td>PEI SN</td>
<td>14.3</td>
<td>396</td>
<td>480</td>
<td>1.21</td>
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<tr>
<td>DE52</td>
<td>90</td>
<td>377</td>
<td>894</td>
<td>2.37</td>
<td>1.16</td>
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</tr>
<tr>
<td>S-200 + amicon</td>
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<td>22</td>
<td>197</td>
<td>8.95</td>
<td>4.37</td>
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E. coli is a great advantage to mechanistic work. The availability of purified apo-E12 enzyme alleviates many problems for studies of cofactor binding, kinetics, and function. The recombinant expression system described herein has allowed us to estimate a $K_m$ of $6.6 \text{ mM}$ for adenosylcobalamin. This value is somewhat surprising, as the enzyme has been described as resisting a litany of harsh methods to remove corrinoids, and has in our hands retained its red color upon precipitation with perchloric acid and trichloroacetic acid, and also after NaOH/SDS denaturation followed by precipitation with potassium acetate at pH 4.8. The measured $K_m$ may only represent an average value of more complex binding and turnover kinetics in this enzyme. Future structural and mechanistic work will certainly address this issue in detail.

Several possible functional interpretations of the Cys-$X_2$-Cys-$X_3$-Cys sequence motif in the $\alpha$-subunit have been eliminated in this study. The sequence appears similar to the structural zinc site in alcohol dehydrogenase, but inconsistent with a catalytic zinc site based on sequence comparisons. Upon removal of metals from KamDE, no significant difference in activity or thermal stability was seen, arguing against the presence of a structural metal site. If present, the stabilizing effect of a metal ion is minimal. The sequence also resembles an iron-sulfur binding site sequence in the corrinoid-iron-sulfur protein. However, 5,6-aminomutase purified from C. sticklandii has never shown any spectroscopic evidence of an iron-sulfur center. Multiple thiols in close proximity could conceivably have a redox function; however, at present the cysteine cluster in KamDE is of unknown function.

Unlike the reports in which $E_2$ was present (3), we have not observed ATP-dependent allostery with purified recombinant KamDE. $E_2$ is known to form a complex with and transfer radioactivity from [8-$^{14}$C]ATP to KamDE. It is likely that the E1-E2 complex exhibits allostery in the aminomutase reaction through the ATP binding function of $E_2$, a similar proposal was made previously by Baker and Stadtman (6). In very recent work on a superficially similar activating enzyme in the diol dehydrase system, ATP was demonstrated to be required for the exchange of free AdoCbl for bound, inactive cobalamins (20). The $\delta$-lysine 5,6-aminomutase system may provide further evidence for such ATP-linked cofactor exchange in the cell, or provide a new paradigm, detailed characterization of $E_2$s.
activating effects on the 5,6-aminomutase reaction awaits further study.

The apparent base-off AdoCbl binding of KamDE is in keeping with a trend observed in other coenzyme B_{12}-dependent enzymes. Enzymes that initiate catalysis by abstracting a hydrogen atom directly from a heteroatom or a carbon bonded to a heteroatom, e.g. ribonucleotide reductase (21), diol dehydrase (22), and ethanolamine ammonia lyase (23), bind AdoCbl with the constituent dimethylbenzimidazole base bonded to the cobalt atom as a lower axial ligand. This binding is therefore referred to as “base-on.” On the other hand, enzymes that abstract hydrogen from a carbon atom without substituents containing electron lone pairs, e.g. glutamate mutase (24) and methylmalonyl-CoA mutase (11), seem to provide a histidine lower axial ligand to the cobalt atom while displacing the dimethylbenzimidazole moiety. The postulated mechanism for KamDE begins by hydrogen abstraction from the δ-carbon of D-lysine, which has only hydrocarbon substituents and falls into the base-off class based on the observed pattern above. Indeed, we have shown directly by EPR spectroscopy that the enzyme binds AdoCbl base-off, consistent with the postulated mechanism.

The experiments described herein initiate a more complete characterization of the enzyme and its reaction mechanism. We believe that D-lysine 5,6-aminomutase may work by a radical mechanism similar to that of L-lysine 2,3-aminomutase (8). The reactions of the two enzymes are very similar. Furthermore, an analogy can be found between their respective cofactors. The putative 5'-deoxyadenosyl radical-generating function of S-adenosylmethionine and a [4Fe-4S] cluster in the 2,3-aminomutase is also associated with adenosylcobalamin. Moreover, both enzymes require pyridoxal phosphate. The chemical rationale for this difference in radical-generating cofactors between the two enzymes is unknown at present.

REFERENCES
Cloning, Sequencing, Heterologous Expression, Purification, and Characterization of Adenosylcobalamin-dependent Lysine 5,6-Aminomutase from *Clostridium sticklandii*

Christopher H. Chang and Perry A. Frey

doi: 10.1074/jbc.275.1.106

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