Identification of Lysine 346 as a Functionally Important Residue for Pyridoxal 5′-Phosphate Binding and Catalysis in Lysine 2,3-Aminomutase from *Bacillus subtilis*†

Dawei Chen and Perry A. Frey*

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

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ABSTRACT: Lysine 2,3-aminomutase (LAM) catalyzes the interconversion of L-lysine and L-β-lysine. The enzyme contains pyridoxal 5′-phosphate (PLP) and a [4Fe-4S] center and requires *S*-adenosylmethionine (SAM) for activity. The hydrogen transfer is mediated by the 5′-deoxyadenosyl radical generated in a reaction of the iron−sulfur cluster with SAM. PLP facilitates the radical rearrangement by forming a lysine−PLP aldimine, in which the imine group participates in the isomerization mechanism. We here report the identification of lysine 346 as important for PLP binding and catalysis. Reduction of LAM with $NaBH_4$ rapidly inactivated the enzyme with concomitant UV/visible spectrum changes characteristic of reduction of an aldimine formed between PLP and lysine. Following reduction with $NaBH_4$ and proteolysis with trypsin, a single phosphopyridoxyl peptide of 36 amino acid residues was identified by reverse-phase liquid chromatography/mass spectrometry (LC/MS). The purified phosphopyridoxyl peptide exhibited an absorption band at 325 nm, and its identity was further confirmed by tandem mass spectrometry (MS/MS) sequencing. The bound PLP is linked to lysine 346 in a PGGGGK (PLP) structure. The sequence of this binding motif is conserved in LAMs from *Bacillus* and *Clostridium* and other homologous proteins but is distinct from the PLP-binding motifs found in other PLP enzymes. The function of lysine 346 was further studied by site-directed mutagenesis. The purified K346Q mutant was inactive, and its content of PLP was only ∼15% of that of the wild-type enzyme. The data indicate that the formation of the aldimine linkage between lysine 346 and PLP is important for LAM catalysis. Sequences similar to the PLP-binding motifs in other enzymes were also present in LAM. However, lysine residues within these motifs neither are the PLP-binding sites in LAM nor are directly involved in LAM catalysis. This study represents the first comprehensive investigation of PLP binding in a SAM-dependent iron−sulfur enzyme.

Lysine 2,3-aminomutase (LAM)[1] catalyzes the interconversion of L-lysine and L-β-lysine, which involves migration of the α-amino group to the β-carbon with the concomitant transfer of the β-hydrogen to the α-carbon. The enzyme contains PLP and [4Fe-4S] clusters and is activated by SAM. The enzyme was first found to participate in the utilization of lysine as carbon and nitrogen sources for the growth of the anaerobic bacterium *Clostridium subterminale* SB4 (*1*−*3*). β-Lysine is also a precursor in the biosynthesis of several antibiotics in *Streptomyces*, such as streptothricin and viomycin (*4, 5*).

The *yodO* gene product of *Bacillus subtilis* was recently identified as a new member of the LAM family (*6*). The enzyme has been characterized as a tetrameric protein with an overall molecular mass of 216 kDa. The DNA-deduced molecular mass of the subunit was confirmed by electrospray ionization mass spectrometry (ESI/MS). Metal and cofactor analyses as well as low-temperature EPR were consistent with the presence of four PLP and two [4Fe-4S] clusters per tetramer. LAM from *Bacillus* is remarkably stable in the air, making it potentially useful for applications in the synthesis of pharmaceutical agents with antibiotic properties.

In analogy to the adenosylcobalmin-dependent rearrangements, the 1,2-hydrogen transfer and 1,2-amino group migration in the reaction of LAM are mediated by the 5′-deoxyadenosyl radical moiety. But unlike the adenosylcobalmin-dependent enzymes, LAM does not contain adenosylcobalmin. Instead, the enzyme is activated by SAM (*2, 7, 8*). Formation of the 5′-deoxyadenosyl radical is brought about by the transient and reversible cleavage of SAM (*9*). The [4Fe-4S] clusters function in providing the electron required for the reductive cleavage of SAM (*10*).

The cleavage of a strong C−S bond by reaction of SAM with an unusual [4Fe-4S] to generate the 5′-deoxyadenosyl radical is unique. Increasing numbers of [4Fe-4S]/SAM enzymes have been identified with information generated from genomic searches. These enzymes function in a diverse range of metabolic pathways from bacteria to mammals. For

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* Correspondence should be addressed to this author at the University of Wisconsin−Madison, 1710 University Ave., Madison, WI 53705. Tel: (608) 262-0055. Fax: (608) 265-2904. E-mail: frey@biochem.wisc.edu.

[1] Abbreviations: LAM, lysine 2,3-aminomutase; SAM, *S*-adenosyl-L-methionine; PLP, pyridoxal 5′-phosphate; HPLC, high-performance liquid chromatography; LC/MS, reverse-phase liquid chromatography/mass spectrometry; EIS/MS, electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; Epps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; BSA, bovine serum albumin.

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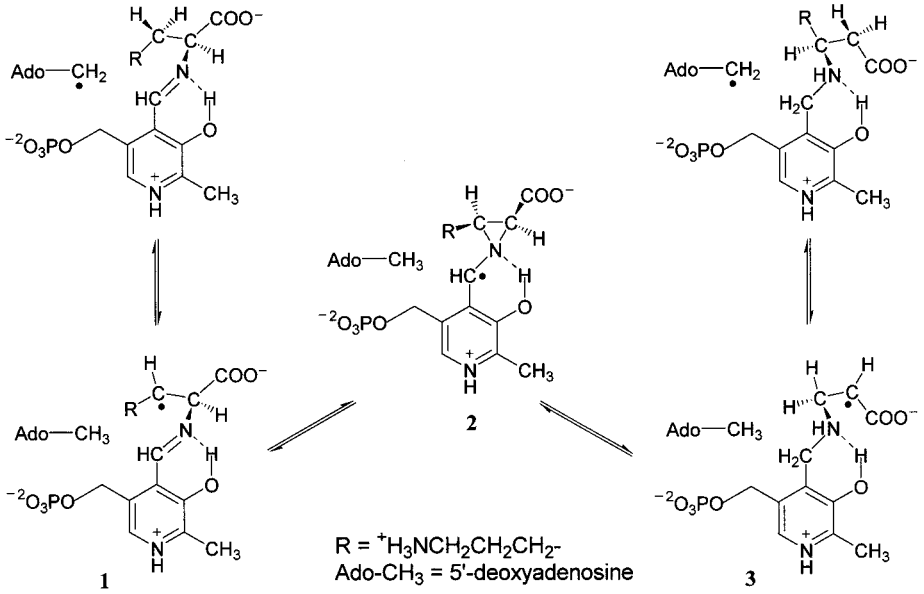
Scheme 1



this reason and for the highly specific and difficult chemical transformations the 5′-deoxyadenosyl radical mediates, the [4Fe-4S]/SAM system occupies an interesting niche in biological chemistry. Among the numerous [4Fe-4S]/SAM enzymes studied in the past decade, the clostridial LAM remains the best characterized.

The radical rearrangement mechanism proposed for the clostridial LAM (*9*−*11*) is illustrated in Scheme 1, in which the 5′-deoxyadenosyl radical initiates the rearrangement by abstracting a hydrogen atom from C-3 of L-lysine to form the substrate-based radical **1**. PLP facilitates the rearrangement by forming an external aldimine linkage to the migrating nitrogen (*12*, *13*) and allowing the transformation of radical **1** into the product-related radical **3**, presumably through the intermediate formation of **2**. Evidence that PLP facilitates α,*β*-imino rearrangements of radical intermediates was obtained from an ESEEM spectroscopic study of the clostridial LAM (*14*). The distance of <3.5 Å measured between the ^{2}H at C4′ of PLP and the radical center at C_α of *β*-lysine was consistent with an aldimine linkage between the bound PLP and the *β*-nitrogen of *β*-lysine. PLP-mediated α,*β*-imino rearrangements of radical intermediates were also supported by nonenzymatic model reaction of *N*-benzylidene-(2-bromomethyl)-DL-alanine ethyl ester with tributyltin hydride under free radical initiation conditions (*15*).

PLP-dependent enzymes catalyze extraordinarily diverse reactions in the metabolism of amino acids, including transamination, decarboxylation, racemization, *β*-elimination, *β*,*γ*-replacement, and many other amino acid reactions (*16*, *17*). In almost all known cases, the PLP binds to the active site by forming an aldimine linkage between the carbonyl group of the cofactor and the *ϵ*-amino group of an active site lysine. In the course of catalysis, the amino group of the substrate replaces the *ϵ*-amino group, and the protonated imine and the pyridine moiety of PLP promote the various chemical transformations through the stabilization of amino acid carbanions.

In the reaction of LAM, the function of PLP appears to be atypical, since the reaction proceeds through a radical rearrangement mechanism. The PLP facilitates the isomerization of radical intermediates, rather than stabilizing carbanions. Notwithstanding the elegance of the proposed mechanism for the LAM reaction, the mode of PLP binding at the active site is not clear. The question of whether an active site lysine is involved in PLP binding through the formation of an aldimine linkage remains to be answered. Amino acid sequence analyses revealed stretches of LAM sequences similar to the PLP-binding motifs found in other PLP-dependent enzymes. Whether these hypothetical motifs constitute the PLP-binding sites in LAM is not known.

In this paper, we report biochemical and biophysical experiments leading to the identification of an active site lysine residue important for PLP binding and catalysis. We present evidence that the hypothetical motifs similar to the PLP-binding sites of other PLP-dependent enzymes do not constitute the PLP-binding sites in LAM and are not directly involved in LAM catalysis.

MATERIALS AND METHODS

Chemicals. L-Lysine was obtained from FisherBiotech. SAM and PLP were from Sigma. SAM was purified by chromatography through a 2 × 20 cm column of CM-cellulose eluted with 40 mM H_2SO_4. Purified SAM was stored at −70 °C. Sodium borohydride, iron standard solution, and spectrophotometric grade trifluoroacetic acid were from Aldrich. HPLC grade acetonitrile was from Fisher Scientific. L-[^{14}C]Lysine was obtained from NEN Life Sciences Products. Sodium boro[^{3}H]hydride was obtained from American Radiolabeled Chemicals. Sequencing grade trypsin was obtained from Promega. Recombinant LAM from *Bacillus subtilis* was expressed and purified as described (*6*). In the preparation of LAM for use in PLP labeling, PLP was omitted from buffers in the DEAE-Sephacel chromatography to minimize adventitiously bound PLP. LAM so purified was designated as enzymes purified in the absence of PLP.

Enzyme Assays and Cofactor Analyses. Protein concentrations were determined using the BCA reagents (Pierce) with BSA as standard. LAM activation and assays were performed

under anaerobic conditions in a Coy anaerobic chamber as previously described (*6*). In $NaBH_4$ inhibition assays, $NaBH_4$ was added to the activated enzyme at the end of reductive incubation at a $NaBH_4$:enzyme molar ratio = 12.5:1 before adding L-lysine, sodium dithionite, and SAM to start the catalytic reaction. One milliunit of the enzyme activity was defined as the amount of enzyme giving rise to 1 nmol of β-lysine per minute under standard assay conditions. For measurements of PLP content, wild-type and mutated proteins of known concentrations were denatured with 0.4 N perchloric acid, and precipitates were removed by centrifugation. The PLP released into solution was analyzed by reaction with phenylhydrazine reagent (*18*). Inorganic sulfide analyses for the purified enzymes were carried out by the method of Beinert (*19*). Iron content was measured as described (*20*).

Enzyme Reduction by Sodium Borohydride. Stock 10 mM $NaBH_4$ in 0.24% NaOH solution was freshly prepared just before use. The PLP form of LAM was reduced by mixing 400 μL of enzyme in 150 mM Epps buffer at pH 8.0 with 100 μL of 10 mM $NaBH_4$. The final enzyme concentration was 100 μM, and the final $NaBH_4$ concentration was 2 mM. In the radioactive labeling experiments, NaB^3H_4 was used. The radioactivity in 100 μL of 10 mM NaB^3H_4 in 0.24% NaOH was 350 μCi. The reduction mixture was incubated at room temperature for 30 min after addition of $NaBH_4$ and then dialyzed against 4 × 2 L of 30 mM Epps buffer at pH 8.0.

Trypsin Digestion. Cysteine residues of the $NaBH_4$-reduced enzyme and the nonreduced control enzyme were carboxymethylated using the method of Imoto and Yamada (*21*). At the end of the alkylation reaction, excess reagents were removed by dialysis against 3 × 2 L of 50 mM NH_4HCO_3 at pH 7.8, and the protein was concentrated using a Centricon 30 concentrator. The concentrated protein was then denatured by heating with 8 M urea in 50 mM NH_4HCO_3 at pH 7.8 in the presence of 5 mM β-mercaptoethanol at 95 °C for 15 min. After cooling to room temperature and diluting the urea to less than 1 M with 50 mM NH_4HCO_3 at pH 7.8, trypsin was added to a final protease:LAM ratio of 1:50 (w/w). The digestion proceeded at 37 °C for 3 h, and an equal amount of trypsin was then added. After 6 h, the pH of the digestion mixture was lowered below 4 with trifluoroacetic acid to stop the proteolysis reaction. Any undissolved materials were removed by centrifugation. Peptides from the trypsin proteolysis were then dried down with a SpeedVac in a vacuum. The dried peptides were redissolved in 0.1% trifluoroacetic acid.

Peptide Mapping. Peptides from the trypsin digestion were separated on a Waters C_{18} column (YMC-Pack ODS-AMQ, S-5 μM, 4.6 × 250 mm) in a gradient of 0−80% acetonitrile (v/v) in 0.1% trifluoroacetic acid at a flow rate of 1 mL min^{-1}. Elution was monitored at both 214 and 274 nm, and fractions of interest were collected and vacuum-dried. Samples were redissolved in 0.1% trifluoroacetic acid for mass spectrometric analyses or in 20 mM Epps buffer at pH 8.0 for spectrophotometric analyses, respectively.

Spectrometry. UV/visible spectra were obtained on a model 8452A diode array spectrophotometer (Hewlett-Packard). Mass spectra were obtained on a Perkin-Elmer Sciex API 365 triple quadrupole ESI mass spectrometer at the University of Wisconsin Biotechnology Center (Madison, WI). LC/MS was performed on the same mass spectrometer equipped with an Applied Biosystem HPLC unit with a Vydac reverse-phase C_{18} column with the detection wavelength at 215 nm. Peptides were eluted into the mass spectrometer with a 90 min gradient consisting of 0−80% acetonitrile in 0.1% trifluoroacetic acid. Peptide sequencing was performed by tandem mass spectrometry (MS/MS). The parent peptide ion was selected with the first quadrupole mass analyzer and transmitted into a collision cell where fragmentation was induced by collision with nitrogen molecules. The collision energy was individually tuned to optimize the quality of the collision spectra. The resulting fragment ions were detected and analyzed with the third mass analyzer.

Table 1: Specific Activities and Contents of PLP, Iron, and Inorganic Sulfide of the Wild-Type and Mutated Lysine 2,3-Aminomutases from *Bacillus*

LAM	activity[a] (milliunits/mg)	PLP[b]	iron[c]	sulfide[d]
wild type	650 ± 50[e]	4.20 ± 0.32	8.40 ± 0.87	7.31 ± 0.56
K361Q	33 ± 4	3.54 ± 0.31	2.78 ± 0.37	2.78 ± 0.37
K290Q	19 ± 3	1.91 ± 0.23	2.11 ± 0.14	1.28 ± 0.21
K346Q	0 ± 0	0.69 ± 0.04	4.37 ± 0.41	3.65 ± 0.22

[a] Activity assays were carried out under standard conditions in Epps buffer at pH 8.0 as described (*6*). [b] PLP was measured by the phenylhydrazine method of Wada and Snell (*18*). [c] Iron was measured as described (*20*). [d] Sulfide was measured by the method of Beinert (*19*). All values are quoted in moles per mole of tetrameric enzyme and are shown by means ± SD.

Site-Directed Mutagenesis. Mutagenic oligonucleotide primers were ordered from Life Sciences Technologies. Site-directed mutagenesis reactions were carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. Mutated genes were completely sequenced using the automated ABI Primer Dye Terminator Cycle Sequencing procedure (University of Wisconsin Biotechnology Center, Madison, WI) to ensure that only the desired mutations were introduced. Plasmid DNAs containing the mutated genes were transformed into BL21(DE3) *E. coli* cells for overexpression as described (*6*).

RESULTS AND DISCUSSION

Site-Directed Mutagenesis of Potentially Important Lysine Residues. In almost all known PLP enzymes, the coenzyme binds to the active site through the formation of an aldimine linkage between the carbonyl group of PLP and the ϵ-amino group of a lysine residue. A PLP-binding motif commonly found in PLP-dependent enzymes is S-X-X-K(PLP) (*22, 23*). The S-X-X-K motif was also found in LAMs from *B. subtilis* and *C. subterminale* (*24*). The amino acid sequence patterns surrounding the S-X-X-K motif in LAMs are similar to the PLP-binding sites of several mitochondrial aspartate aminotransferases (*25*−*28*). In addition to S-X-X-K, nearby residues at the N-terminal side of the motif are well conserved between aminotransferases and the corresponding LAM sequences.

This hypothetical PLP-binding site was investigated by site-directed mutagenesis of lysine 361 in the *Bacillus* LAM, which was replaced with a glutamine residue. The catalytic activity and coenzyme contents of the mutated enzyme were evaluated. Table 1 shows the comparisons of enzyme activity and coenzyme contents of *Bacillus* K361Q-LAM with those

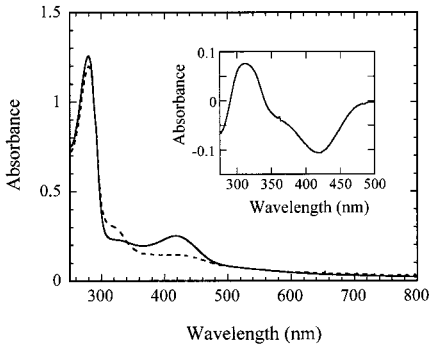


FIGURE 1: Spectral changes upon reduction of LAM by sodium borohydride. The UV/visible spectrum of 0.15 mg of *Bacillus* LAM purified in the absence of PLP in 100 mM Epps buffer at pH 8.0 (solid line) was obtained. After addition of 10 μL of 20 mM $NaBH_4$, the mixture was incubated at room temperature for 5 min, and the spectrum of the reduced enzyme was taken (dashed line). The difference spectrum of the reduced and the unreduced enzymes is shown in the inset, which highlights the absorption decrease at the 420 nm region and the increase at the 325 nm region upon reduction. The spectral change is consistent with reduction of an aldimine linkage between the bound PLP and a lysine residue.

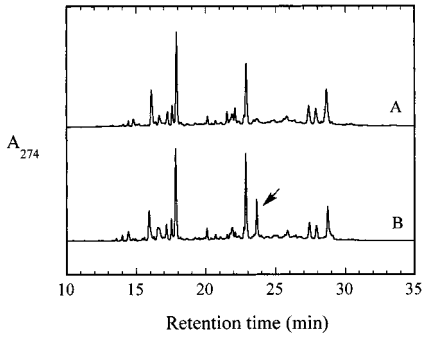


FIGURE 2: Fractionation of peptides derived from trypsin digestion of the LAM peptides. Samples of $NaBH_4$-reduced and unreduced LAM were subjected to trypsin digestion and chromatographed on a reverse-phase C_{18} HPLC column (see Materials and Methods for details). Fractions were monitored at 274 nm for clarity in the chromatograms shown. A fraction emerged at ~23.7 min, as marked by an arrow in panel B, and was derived from proteolysis of the $NaBH_4$-reduced enzyme. This fraction was absent in peptides derived from proteolysis of the unreduced enzyme (panel A). The 23.7 min fraction is a candidate for the localization of the PLP-binding site.

of the wild-type enzyme. The PLP content of K361Q-LAM was comparable to that of the wild-type enzyme, while its activity was ~5% of that of the wild-type enzyme. The high content of PLP and the retention of very substantial activity show that lysine 361 is not absolutely required for PLP binding and catalysis. Interestingly, the contents of Fe and inorganic sulfide were low in K361Q-LAM. The loss of Fe and sulfide at least partially accounts for the low activity.

Sequence analyses also revealed LAM sequences similar to the PLP-binding motifs found in serine and threonine dehydratases (*29*). The *Bacillus* and clostridial LAMs retain the K-X-R motif of the PLP-binding sites found in four serine and threonine dehydratases. The possible role of this LAM motif in binding PLP was studied by replacing lysine 290 of the *Bacillus* LAM with glutamine. The activity and the PLP, iron, and sulfide content were analyzed. The results are included in Table 1. The activity of K290Q-LAM was ~30-fold less than that of the wild-type enzyme, while the PLP content was ~50% of that of the wild-type enzyme. The changes in activity and PLP content are not comparable. However, similar to the K361Q-LAM, the contents of iron and sulfide were significantly affected. The retention of partial activity and partial PLP binding capability suggests that lysine 290 is not involved in PLP binding and catalysis.

Sodium Borohydride Reduction of Bacillus LAM. The UV/visible spectrum of wild-type LAM consists of absorption bands in both the UV and the visible regions (Figure 1, solid line). The absorption bands of the visible region are due to the [4Fe-4S] cluster and PLP. The prominent peak at 420 nm at pH 8.0 is characteristic of aldimine formation between the bound PLP and the ϵ-amino group of a lysine.[2]

The 420 nm absorption band is bleached by addition of sodium borohydride to the enzyme solution, concomitant with an absorption increase at 325 nm (Figure 1, dashed line and inset). The spectral changes are consistent with reduction of an aldimine linkage between PLP and a lysine residue. In addition to the spectral changes, $NaBH_4$ treatment inhibits LAM activity. The activity decreased more than 20-fold when the reductively activated enzyme was treated with a 12.5-fold molar excess of $NaBH_4$ (data not shown). The loss of enzyme activity upon reduction by $NaBH_4$ suggests a functional role of the aldimine linkage between PLP and the lysine residue.

Identification of Peptide with the PLP-Binding Site. The aldimine linkage, as suggested by the spectral change upon $NaBH_4$ reduction of the enzyme, could be formed either between PLP and a protein lysine residue or between PLP and the bound substrate at the active site. To identify this lysine and answer the question of whether an active site lysine is involved in PLP binding in LAM, samples of $NaBH_4$-reduced LAM and unreduced enzyme were carboxymethylated, denatured, and digested with trypsin, respectively. After denaturation and proteolysis, peptides from the $NaBH_4$-reduced enzyme retained the 325 nm absorption band in addition to the protein band at 280 nm (data not shown). By contrast, the 325 nm absorption band could not be observed in peptides from the unreduced enzyme, indicating the loss of PLP from the unreduced enzyme during denaturation and dialysis in the carboxymethylation step. Taken together, the data suggested that the lysine involved in aldimine formation was an intrinsic protein lysine residue.

To identify this possible active site lysine residue, peptides from the $NaBH_4$-reduced and unreduced enzymes were separately fractionated by HPLC on a C18 reverse-phase column, and the chromatograms were compared (Figure 2). In the HPLC of peptides from the $NaBH_4$-reduced enzyme, a fraction emerged at 23.6−23.8 min (Figure 2, B), and was absent in the HPLC of peptides from the unreduced enzyme (Figure 2, A). The 23.7 min fraction could be visualized when the elution was monitored at either 214 or 274 nm, suggesting the presence of aromatic residues in this fraction. When NaB^3H_4 was used in enzyme reduction, this fraction

[2] Initial experiments with $NaBH_4$ reduction carried out on clostridial LAM did not give promising results because of the difficulty of completely inactivating the enzyme with $NaBH_4$. Therefore, the pursuit of the PLP-binding site in the *Bacillus* enzyme, which is less oxygen-sensitive and more easily purified than the clostridial LAM, was initiated by mutagenesis of lysine residues in sequence motifs related to other PLP enzymes. The unsatisfactory results reported herein led to a reexamination of $NaBH_4$ reduction of the *Bacillus* LAM, which proved to be completely inactivated by high concentrations of borohydride.

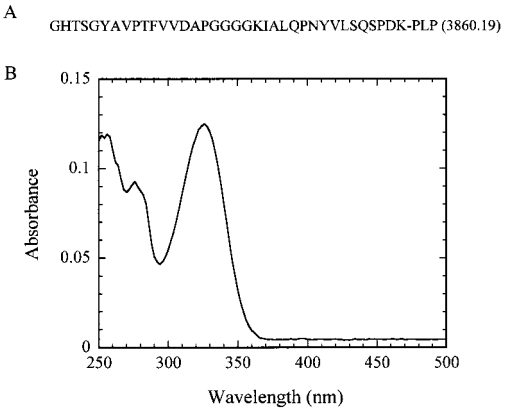


FIGURE 3: PLP-labeled peptide identified by $NaBH_4$ reduction, peptide mapping, ESI/MS, and LC/MS. The amino acid sequence and the calculated mass value of the PLP-modified peptide are shown in panel A. The mass of this peptide measured by LC/MS and ESI/MS was 3860.11 ± 0.18 and 3860.64 ± 0.26 Da, respectively, which equals the calculated mass of the peptide (3629.05 Da) plus the masses of PLP (247.14 Da) and two hydrogen atoms (2.016 Da) minus the mass of H_2O (18.016 Da). The extra 231 mass units were due to the attachment of the PLP moiety. The absorption spectrum of the PLP-labeled peptide is shown in panel B. The 325 nm absorption band indicates a reduced aldimine linkage between the aldehyde group of PLP and the ϵ-amino group of the active site lysine residue.

contained more than 85% of the total radioactivity associated with the protein (data not shown), indicating that the active site lysine was located within this peptide. The 23.7 min peptide was collected and subjected to further analyses by ESI/MS and UV/visible spectrophotometry. In the ESI/MS analysis, the molecular mass of the isolated peptide was found to be 3860.64 ± 0.26 Da. It equals the mass of a 36 residue peptide (3629.05 Da) from glycine 326 to lysine 361 of *Bacillus* LAM, plus 231.14 mass units due to the attachment of the PLP moiety (see panel A of Figure 3 for the sequence of the peptide). Furthermore, the 23.7 min fraction exhibits an absorption band at 325 nm at pH 8.0 (Figure 3, panel B) in addition to the 274 nm band. The 274 nm band is mainly due to the presence of two tyrosine residues in the peptide, while the 325 nm band clearly indicated a reduced aldimine linkage between PLP and the ϵ-amino group of a lysine residue within this peptide.

To further investigate the identity of this lysine residue, peptides from the $NaBH_4$-reduced enzyme and the unreduced enzyme were analyzed by LC/MS. In the LC/MS of peptides from the $NaBH_4$-reduced enzyme, a peptide of 3859.98 ± 0.18 Da was identified. This peptide could not be identified in the LC/MS analyses of peptides of the unreduced enzyme. Instead two species of 1974.36 ± 0.41 and 1672.22 ± 0.30 Da were present in peptides from the unreduced enzyme, which were absent in the peptides of the $NaBH_4$-reduced enzyme. The peptides of 1974.36 ± 0.41 and 1672.22 ± 0.30 Da from the unreduced enzyme correspond to the masses of peptides 326 GHTSGYAVPTFVVDAPGGGGK 346 and 347 IALQPNYVLSQSPDK 361, respectively, which are the two halves of the 36 residue peptide. The absence of these two species in the LC/MS of peptides from the $NaBH_4$-reduced enzyme suggests that lysine 346 at the center of the peptide was covalently modified by PLP, which renders resistance to trypsin cleavage at this position.

MS/MS Sequencing of the PLP-Labeled Peptide. As can be seen from Figure 3, the identified phosphopyridoxyl peptide contains two lysine residues. Lysine 346 is near the center of the peptide and lysine 361 at the C-terminal end. The resistance to trypsin cleavage at the central lysine suggests that PLP binds to lysine 346. Evidence that PLP indeed binds to lysine 346 but not lysine 361 was obtained through peptide sequencing of the 3861 Da peptide by MS/MS. The parent ion of 3861 Da was selected and subjected to collision-induced fragmentation, and masses of the fragment ions were analyzed. The mass data of fragment ions observed in the MS/MS of the 3861 Da peptide are summarized in Figure 4.

For the two groups of fragment ions generated, ions with the N-terminal glycine are shown above the parent peptide and those with the C-terminal lysine below the parent peptide. Masses of the parent phosphopyridoxyl peptide (3629 + 231) and those peptide ions with an additional 231 mass units due to the attachment of the PLP moiety are indicated. The sequencing data indisputably prove that PLP binds to lysine 346 of the *Bacillus* LAM, and an internal aldimine linkage is indeed formed between the bound PLP and lysine 346 at the active site. The sequencing data also provided an explanation for why trypsin failed to cleave at lysine 346 of the $NaBH_4$-reduced enzyme. The MS/MS fragment masses also further confirmed the identity of the PLP-labeled peptide identified by ESI/MS and LC/MS.

Examining the amino acid sequences of LAMs and six of their homologues (*24*), it is noted that the active site lysine identified in this paper is fully conserved in all of the sequences (Figure 5). These sequences share overall sequence identities to the clostridial LAM ranging from 32 to 72% (*24*). The high amino acid sequence homologies suggest that they share a similar three-dimensional structure. The presence of the newly identified PLP-binding motif, i.e., P-G-G-G-G-K, in all these sequences suggests that they may all be PLP-dependent and belong to the same [4Fe-4S]/SAM aminomutase family. The P-G-G-G-G-K motif of LAM was also found to exist in the human Lasp-1 protein of unknown function (*30*), although Lasp-1 does not share a significant overall sequence homology with LAMs. Interestingly, Lasp-1 was found to be overexpressed in some breast carcinomas.

Site-Directed Mutagenesis of Lysine 346. To further investigate whether an internal aldimine formation between PLP and the active site lysine is absolutely required for PLP binding and catalysis, site-directed mutagenesis was performed to replace lysine 346 with a glutamine residue. In the purification of K346Q-LAM, no abnormal elution behaviors were observed in either the DEAE-Sephacel ion exchange chromatography or the Sephacryl S-300 gel filtration chromatography, suggesting the lysine 346 to glutamine change did not cause a global structural perturbation of the enzyme. Activity assays using L-[^{14}C]lysine indicated that K346Q-LAM was inactive. Formation of L-β-lysine could not be detected even in assays with prolonged incubation. The PLP content of the purified K346Q-LAM was only about 15% of the wild-type enzyme (Table 1). Substitution of lysine 346 with glutamine apparently caused a dramatic decrease in PLP-binding affinity and resulted in a significant loss of the bound coenzyme. The small amount of PLP present in the mutant enzyme may represent some nonproductively bound PLP. The low content of PLP and lack of catalytic activity indicate that lysine 346 is a functionally important residue; PLP binds to the active site by forming an internal

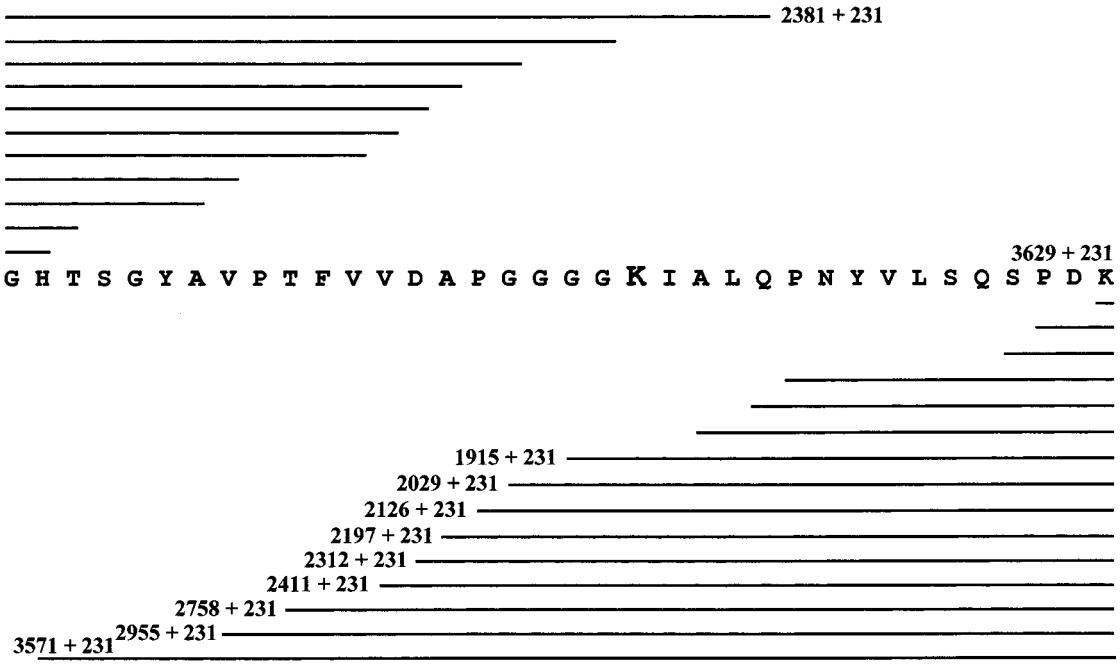


FIGURE 4: MS/MS sequencing of the 36 residue peptide of 3860 Da. The amino acid sequence of the 3860 Da peptide is shown, with the putative active site lysine shown in boldface. The fragmented peptide ions with the N-terminal glycine are shown above the parent peptide ion and those with the C-terminal lysine below the parent peptide. The masses of the parent phosphopyridoxyl peptide (3629 + 231) and those fragment peptides with an additional 231 mass units due to the attachment of the PLP moiety are indicated.

339	DAPGGGG**K**IALQP	351	*B. subtili* LAM
	DAPGGGG**K**IPVMP		*P. gingivalis*
345	DAPGGGG**K**TPVMP	357	*C. subterminale* LAM
	DAPGGGG**K**IPVAP		*D. radiodurans*
	DLPGGKG**K**VPLLP		*A. aeolicus*
	DLPGGGG**K**FPLVA		*T. pallidum*
	EIAGEPN**K**TLYAE		*H. influenzae*
	EIGGEPS**K**TPLDL		*E. coli*

FIGURE 5: PLP-binding site identified in LAM from *Bacillus* and the corresponding sequences in its homologues. The active site lysine in the *Bacillus* LAM is residue 346, and in *Clostridium subterminale* LAM it is lysine 337. The other homologues, some of which may be LAMs, retain this lysine residue. The *E. coli* homologue is not a LAM (*24*), and its biological function remains unknown, but it retains the conserved lysine residue.

aldimine linkage with the ϵ-amino group of lysine 346. The internal aldimine linkage facilitates productive PLP binding and catalysis.

Replacement of lysine 346 with glutamine also resulted in significant decreases in iron and sulfide contents (Table 1). The K346Q-LAM contained only ~3.7 sulfide and ~4.4 Fe per tetramer, instead of the usual ~8 Fe and ~8 sulfide per tetramer of the wild-type enzyme. The loss of iron and sulfide apparently resulted from the loss of PLP, which may perturb the active site structure and destabilize the [4Fe-4S] cluster.

The requirement of PLP in the reaction of LAM has been well established (*2*, *12*, *14*, *15*). The involvement of PLP in facilitating rearrangement of radical intermediates was supported by nonenzymatic model reactions as well as by spectroscopic studies. However, the mode of PLP binding at the active site of this SAM-dependent [4Fe-4S] enzyme has not been determined before. During catalysis, the substrate- and product-based radical intermediates are bound to PLP by forming the external aldimine adducts. One pivotal question about PLP binding and catalysis in LAM has been whether it is bound to a lysine residue. And if such an active site lysine exists, whether an internal aldimine linkage is required for productive PLP binding and catalysis. What is its role in substrate binding and product release? And how does it modulate the radical rearrangement? This paper has provided answers to the first two questions above. A lysine residue indeed exists at the active site of LAM. It was localized by $NaBH_4$ reduction, proteolysis of the reduced enzyme, chromatographic fractionation, and UV/visible and mass spectroscopic analyses of the peptide fractions. The active site lysine is located in a distinctive motif highly conserved among LAM homologues, at positions 346 and 337 in the *Bacillus* and clostridial enzymes, respectively. The $NaBH_4$ inhibition of enzyme activity and the spectral changes upon $NaBH_4$ reduction as well as the results of mutagenesis of lysine 346 all indicate the formation of internal aldimine linkage between the bound PLP and the active site lysine. The internal aldimine linkage is important for productive PLP binding and LAM catalysis.

The limitation of sequence analysis in the identification of the PLP-binding site is apparent, especially considering the diverse features of the PLP motifs of the various PLP-dependent enzymes. This study could have some bearing on the identification of the PLP site of newly identified [4Fe-4S]/SAM-dependent aminomutases or other newly identified enzymes involved in amino acid metabolism.

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REFERENCES

1. Costilow, R. N., Rochovansky, O. M., and Barker, H. A. (1966) *J. Biol. Chem. 241*, 1573−1578.

2. Chirpich, T. P., Zappia, V., Costilow, R. N., and Barker, H. A. (1970) *J. Biol. Chem. 245*, 1778−1789.
3. Stadtman, T. C. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 38*, 413−448.
4. Charter, J. H., II, Du Bus, R. H., Dyer, J. R., Floyd, J. C., Rice, K. C., and Shaw, P. D. (1974) *Biochemistry 13*, 1227−1233.
5. Thiruvengadam, T. K., Gould, S. J., Aberhart, J. D., and Lin, H. J. (1983) *J. Am. Chem. Soc. 105*, 5470−5476.
6. Chen, D., Ruzicka, F. J., and Frey, P. A. (2000) *Biochem. J. 348*, 539−549.
7. Petrovich, R. M., Ruzicka, F. J., Reed, G. H., and Frey, P. A. (1992) *Biochemistry 31*, 10774−10781.
8. Moss, M. L., and Frey, P. A. (1990) *J. Biol. Chem. 265*, 18112−18115.
9. Moss, M., and Frey, P. A. (1987) *J. Biol. Chem. 262*, 14859−14862.
10. Lieder, K. W., Booker, S., Ruzicka, F. J., Beinert, H., Reed, G. H., and Frey, P. A. (1998) *Biochemistry 37*, 2578−2585.
11. Baraniak, J., Moss, M. L., and Frey, P. A. (1989) *J. Biol. Chem. 264*, 1357−1360.
12. Frey, P. A. (1993) *FASEB J. 7*, 662−670.
13. Frey, P. A. (1990) *Chem. Rev. 90*, 1343−1357.
14. Ballinger, M. D., Frey, P. A., and Reed, G. H. (1995) *Biochemistry 34*, 10086−10093.
15. Han, O., and Frey, P. A. (1990) *J. Am. Chem. Soc. 112*, 8982−8983.
16. John, R. A. (1995) *Biochim. Biophys. Acta 1248*, 81−96.
17. Walsh, C. (1979) in *Enzymatic Reaction Mechanisms*, pp 777−827, W. H. Freeman & Co, New York.
18. Wada, H., and Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089−2095.
19. Beinert, H. (1983) *Anal. Biochem. 131*, 373−378.
20. Kennedy, M. C., Kent, T. A., Emptage, M., Merkle, H., Beinert, H., and Münck, E. (1984) *J. Biol. Chem. 259*, 14463−14471.
21. Imoto, T., and Yamada, H. (1989) in *Protein Function* (Creighton, T. E., Ed.) p 263, IRL Press, Oxford.
22. Vaaler, G., and Snell, E. E. (1989) *Biochemistry 28*, 7306−7313.
23. Stover, P., Zamora, M., Shostak, K., Gautam-Basak, M., and Schirch, V. (1992) *J. Biol. Chem. 267*, 17679−17687.
24. Ruzicka, F. J., Lieder, K. W., and Frey, P. A. (2000) *J. Bacteriol. 182*, 469−476.
25. Barra, D., Martini, F., Montarani, G., Doonan, S., and Bossa, F. (1979) *FEBS Lett. 108*, 103−106.
26. Graf-Hausner, U., Wilson, K. J., and Christen, P. (1983) *J. Biol. Chem. 258*, 8813−8826.
27. Huynh, Q. K., Sakakibara, R., Watanabe, T., and Wada, H. (1980) *Biochem. Biophys. Res. Commun. 97*, 474−479.
28. Barra, D., Bossa, F., Doonan, S., Fahmy, H. M. A., Hughes, G. J., Martini, F., Petruzzelli, R., and Wittmann-Liebold, B. (1980) *Eur. J. Biochem. 108*, 405−414.
29. Ogawa, H., Konishi, K., and Fujioka, M. (1989) *Biochim. Biophys. Acta 996*, 139−141.
30. Moog-Lutz, C., Regnier, C. H., Schreiber, V., Basset, P., and Rio, M. C. (1995) *FEBS Lett. 373*, 245−249.

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