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Identification of a Novel Pyridoxal 5′-Phosphate Binding Site in Adenosylcobalamin-Dependent Lysine 5,6-Aminomutase from *Porphyromonas gingivalis*†

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ABSTRACT: Lysine 5,6-aminomutase (5,6-LAM) catalyzes the interconversion of D-lysine with 2,5-diaminohexanoate and of L-β-lysine with 3,5-diaminohexanoate. The coenzymes for 5,6-LAM are adenosylcobalamin (AdoCbl) and pyridoxal 5′-phosphate (PLP). In the proposed chemical mechanism, AdoCbl initiates the formation of substrate radicals, and PLP facilitates the radical rearrangement by forming an external aldimine linkage with the ϵ-amino group of a substrate, either D-lysine or L-β-lysine. In the resting enzyme, an internal aldimine between PLP and an essential lysine in the active site facilitates productive PLP binding and catalysis. We present here biochemical, biophysical, and site-directed mutagenesis experiments, which document the existence of an essential lysine residue in the active site of 5,6-LAM from *Porphyromonas gingivalis*. Reduction of 5,6-LAM with $NaBH_4$ rapidly inactivates the enzyme and shifts the electronic absorption band from 420 to 325 nm. This is characteristic of the reduction of an aldimine linkage between the carbonyl group of PLP and the ϵ-amino group of a lysine residue. The reduced peptide was identified by Q-TOF/MS and further confirmed by Q-TOF/MS/MS sequencing. We show that lysine 144 in the small subunit of 5,6-LAM is the essential lysine residue. Lysine 144(β) is separated by only 11 amino acids from histidine 133(β), which forms a part of the "base-off"-AdoCbl binding motif. The sequence of the novel PLP-binding motif is conserved in 5,6-LAM from *Clostridium sticklandii* and *P. gingivalis*, and it is distinct from all known PLP-binding motifs. Mutation of lysine 144(β) to glutamine led to K144Q(β)-5,6-LAM, which displayed no enzymatic activity and no absorption band corresponding to an internal PLP-aldamine. In summary, we introduce a novel PLP-binding motif, the first to be discovered in an AdoCbl-dependent enzyme.

Lysine 5,6-aminomutase (5,6-LAM),[1] first found in *Clostridium sticklandii*, participates in the fermentation of DL-lysine (*1*, *2*). 5,6-LAM catalyzes the migration of the ϵ-amino group of either D-lysine or L-β-lysine to the δ-carbon, with concomitant reverse migration of a hydrogen atom to produce 2,5- or 3,5-diaminohexanoic acid (2,5/3,5-DAH) (*1*, *2*). Both AdoCbl and PLP are essential coenzymes for the action of 5,6-LAM (*1*). The reaction is reversible, and the equilibrium constant (K_{eq}) for the conversion of D-lysine into 2,5-diaminohexanoate is 1.2 (*3*).

The action of 5,6-LAM requires two protein components, the core enzyme E_1 and an activating protein E_2 (*1*). E_1 is a heterotetramer of α and β subunits formulated as $\alpha_2\beta_2$, the molecular mass[2] of which is approximately 170 kDa. The molecular masses of the β and α subunits are 29 and 57 kDa, respectively (*1*, *4*). The amino acid sequences of 5,6-LAM from *C. sticklandii* (*4*) and *Porphyromonas gingivalis* indicate a conserved "base-off/histidine-on" AdoCbl binding motif in the small subunit, as well as a three-cysteine cluster and a consensus P-loop motif in the large subunit. Earlier EPR experiments on 5,6-LAM (*4*) with [^{15}N]dimethylbenzimidazole−AdoCbl demonstrated binding of AdoCbl with [^{15}N]dimethylbenzimidazole not bound to cobalt, as in other B_{12}-dependent enzymes incorporating the "base-off" motif, including glutamate mutase, methionine synthase, methylmalonyl-CoA mutase, and D-ornithine aminomutase (*5*−*9*). In 5,6-LAM, the dimethylbenzimidazole moiety is presumably replaced with His133(β), embedded in the D**H**XXG motif as in other base-off-type AdoCbl proteins.

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[1] Abbreviations: AdoCbl, adenosylcobalamin; PLP, pyridoxal 5′-phosphate; PNP, pyridoxine 5′-phosphate; β-ME, β-mercapoethanol; 5,6-LAM, lysine 5,6-aminomutase; 2,5/3,5-DAH, 2,5/3,5-diaminohexanoic acid; DTT, dithiothreitol; Epps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; EPR, electron paramagnetic resonance spectroscopy; Gdn-HCl, guanidine hydrochloride; HPLC, high-pressure liquid chromatography; LAM, L-lysine 2,3-aminomutase; MS, mass spectrometry; LC/MS, reverse-phase liquid chromatography mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; Micromass Q-TOF2, Micromass hybrid quadrupole/orthogonal time of flight mass spectrometry; MS/MS, tandem mass spectrometry; ESI/MS, electrospray ionization mass spectrometry; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

[2] All the masses (*m*) in this paper represent monoisotopic molecular masses unless otherwise indicated.

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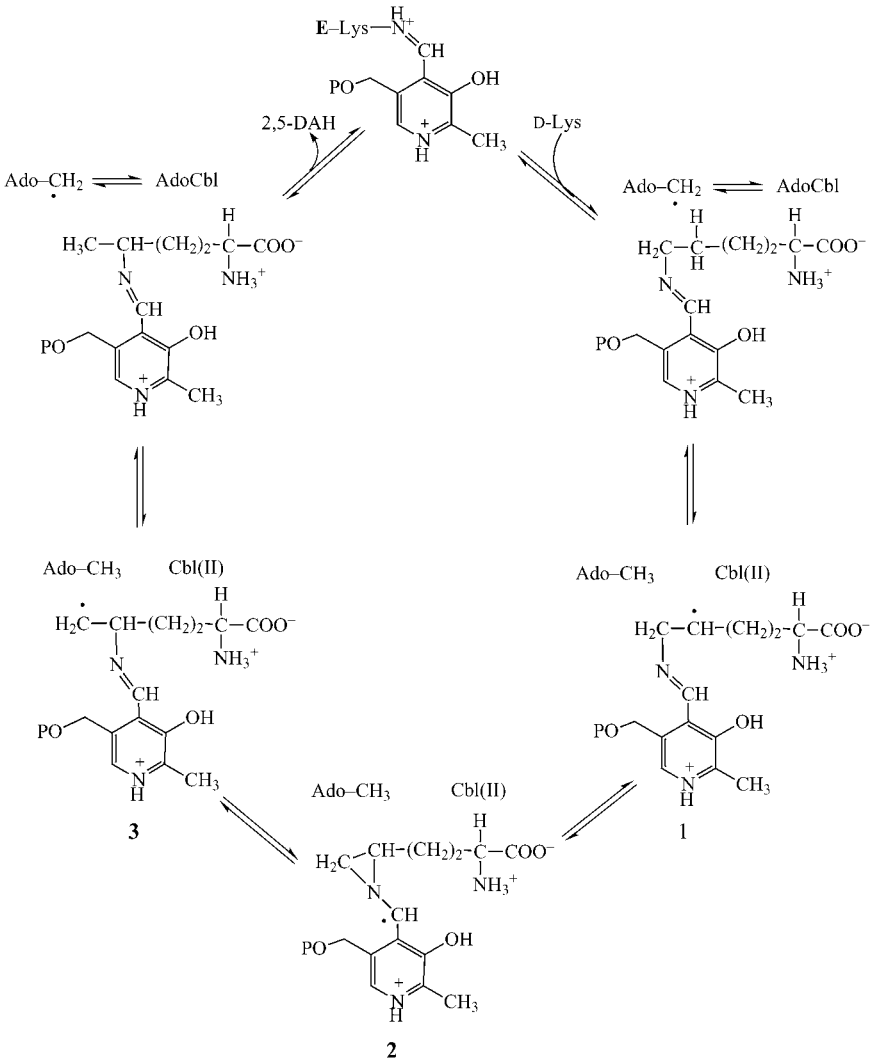

FIGURE 1: Proposed mechanism of action of 5,6-LAM. The mechanism proposed here is the radical rearrangement catalyzed by 5,6-LAM. In this mechanism, PLP binds the substrate as the external aldimine through its ϵ-amino group. Substrate radical formation is brought about by abstraction of the C5(H) from the side chain of lysine by the 5′-deoxyadenosyl radical derived from AdoCbl. Rearrangement of the substrate-related radical **1** to the product-related radical **3** is facilitated by the imine linkage to PLP and the intermediate formation of the azacyclopropylcarbinyl radical **2**. Hydrogen abstraction by radical **2** from the methyl group of 5′-deoxyadenosine, which remains bound to the active site, leads to the external aldimine of 2,5-DAH and PLP. The release of 2,5-DAH is brought about by transaldimination with the active site lysine to regenerate the internal aldimine of PLP.

The mechanism we propose in Figure 1 is analogous to that established for the L-lysine 2,3-aminomutase (LAM) from *Clostridium subterminale* (*11*), except that the function of the [4Fe-4S] cluster and *S*-adenosyl-L-methionine (SAM) in the reaction of LAM in generating the transient 5′-deoxyadenosyl radical is replaced by AdoCbl in the reaction of 5,6-LAM. The radical mechanism for the 1,2-amino group migration has been documented by spectroscopic experiments in the reaction of LAM (*12*) and is the inspiration for the hypothetical mechanism in Figure 1 for the reaction of 5,6-LAM. The reaction begins with transaldimination between D-lysine and the internal PLP-aldimine to form the external D-lysyl-PLP-aldimine. Reversible homolytic scission of the Co−C5′ bond in AdoCbl generates the 5′-deoxyadenosyl radical, which immediately abstracts the C5(H) from the side chain of D-lysine. The resulting radical **1** rearranges by way of the azacyclopropylcarbinyl radical **2** to the product-related radical **3**. Abstraction of a methyl hydrogen from 5′-deoxyadenosine by radical **3** regenerates the 5′-deoxyadenosyl radical and produces the external PLP-aldimine of the product. 2,5-DAH is released upon formation of the internal PLP-aldimine with the active site lysine. In most known PLP-dependent enzymes, conserved lysine residues in PLP-binding motifs can be found in their amino acid sequences. No known PLP-binding motif is found in the sequence of 5,6-LAM from either *C. sticklandii* or *P. gingivalis*.

In this report, we present biochemical, biophysical, and site-directed mutagenesis experiments supporting the presence of an essential lysine residue in the active site of 5,6-LAM from *P. gingivalis*. We show that this novel PLP-binding site is not related to any conserved sequence motif in other PLP-dependent enzymes. However, this same motif is present in the amino acid sequence of 5,6-LAM from *C. sticklandii* and in the homologous amino acid sequences of ornithine 4,5-aminomutase from *Clostridium difficile* and *C. sticklandii*.

MATERIALS AND METHODS

Chemicals. D-Lys-HCl, AdoCbl, Tris, Epps, EDTA, β-mercaptoethanol, TCA, PMSF, and PLP were purchased from

Sigma. Poly(ethylenimine) was purchased from Acros, sodium borohydride and iodoacetic acid were from Aldrich, D-[1-^{14}C]lysine and [U-^{14}C]PLP were from ARC, IPTG was from Inalco Pharmaceuticals, Gdn-HCl was from Life Technologies, kanamycin was from Fisher, chloramphenicol was from Sigma, DEAE-cellulose DE52 was from Whatman, phenyl-Sepharose 6 Fast Flow wasfrom Amersham Pharmacia Biotech, sequencing grade endoprotease Lys-C (Lys-C) was from Roche Molecular Biochemicals, plasmid miniprep kits were from Qiagen, and the micropure-EZ spin column was from Millipore. The *P. gingivalis* W83 genomic DNA was a generous gift from Dr. Margaret Duncan (The Forsyth Institute, Boston, MA).

Enzymes, Bacterial Strain, and Plasmids. Restriction endonucleases were purchased as follows: *Nco*I and *Hin*III were from Promega, *Pfu* DNA polymerase was from Stratagene, and T4 DNA ligase and calf intestine alkaline phosphatase were from Promega. Expression vector pET-28b(+) was purchased from Novagen and pCR-Blunt II TOPO from Invitrogen. Competent *Escherichia coli* cells were purchased as follows: XL-2 Blue MRF′ Ultracompetent and XL-10 Gold ultracompetent were from Stratagene, and Rosetta(DE3) was from Novagen.

Preparation of the E. coli Expression Vector for the 5,6-LAM Gene from P. gingivalis. The *P. gingivalis* 5,6-LAM gene (*KamD* and *KamE*) was amplified from *P. gingivalis* W83 genomic DNA. The one base pair downstream of the *KamD* start codons was mutated in the 5′-primer to give a *Nco*I site, and the 3′-primer contained a *Hin*dIII site downstream of the stop codon for cloning into the expression vector. The 5′-primer had the sequence 5′-TACACCATG-GAAAAAAGTAAAGTCGG-3′ and the 3′-primer had the sequence 5′- TAGAAAGCTTTTATTTGTTATTCATTC-TCTTATGAAACTCC-3′ (restriction site underlined). The PCR reaction mixture contained 1 μg of genomic DNA, 0.2 mM deoxynucleoside triphosphates, 0.5 μM oligonucleotide primers, and cloned *Pfu* DNA polymerase; 5 units in *Pfu* DNA polymerase reaction buffer was supplied by the manufacturer. The sample was subjected to 30 cycles of 1 min at 94 °C, 30 s at 37 °C, 15 s at 50 °C, and 3 min at 72 °C. After thermocycling, the expected 2.4 kb DNA product formed during the PCR process was purified by agarose electrophoresis (2% agarose). The purified PCR product was blunt-end ligated to the pCR-Blunt II-TOPO cloning vector according to the manufacturer's specification. Plasmid DNA was double digested with *Nco*I/*Hin*dIII, and the *KamDE* fragment was gel purified as described before. PET-28b(+) (5 μg) was similarly digested with *Nco*I/*Hin*dIII, dephosphorylated with 1 unit of calf intestinal alkaline phosphatase for 30 min at 37 °C, purified by a Micro-EZ spin column, and ethanol precipitated. *KamDE* and cut pET-28b(+) were ligated with T4 DNA ligase. The ligation mixture was used to transform XL-2 Blue MRF′ ultracompetent cells to kanamycin resistance. Plasmid DNA from a number of transformants was isolated and screened for the insert by restriction digestion with *Nco*I/*Hin*dIII as well as complete sequencing.

DNA Sequencing. The transformant plasmid DNA was performed using materials and protocol from the University of Wisconsin Biotechnology Center, Madison, WI. Reactions were run through an Amerisham Pharmacia Biotech G-50 spin column, dried, and submitted for analysis. The DNA plasmid containing the complete sequence was used to transform Rosetta (DE3) *E. coli* cells for the overexpression of 5,6-LAM.

Site-Directed Mutagenesis. Mutagenic oligonucleotide primers were ordered from the DNA Synthesis Facility, Biotechnology Center, University of Wisconsin, Madison, WI. Site-directed mutagenesis reactions were carried out using the QuikChange XL 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 the desired mutations were introduced. Plasmid DNA containing the mutated genes was transformed into Rosetta (DE3) *E. coli* cells for expression as described in the previous section of wild-type 5,6-LAM.

Expression and Purification of the Recombinant 5,6-LAM. A 1% overnight culture of cells carrying *KamDE* was used to inoculate 1.0 L of LB medium containing kanamycin (50 μg/mL) and grown aerobically to an A_{600} of 0.6−0.7 at 37 °C. Expression was induced by addition of IPTG to a final concentration of 1.5 mM, and growth continued aerobically at 30 °C for 16 h. Cells were harvested by centrifugation at 6500*g*, 4 °C for 20 min. Approximately 2−2.5 g of cells was harvested from a 1.0 L cell culture. The cell pellet was resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM β-ME, with or without 1 mM PLP, 1 mM EDTA, 0.2 mM NaCl, and 0.1% Triton X-100) and sonicated (4 × 40 s at power 6 with a Fisher Model 550 sonic dismembrator). PMSF was added to 1 mM, and the suspension was centrifuged at 12000*g* for 40 min. Poly(ethylenimine) was added to the supernatant fluid to 0.1%, and the suspension centrifuged at 12000*g*, 4 °C for 1 h. The supernatant was diluted to 250 mL with purification buffer, and 5,6-LAM was purified as previously described with minor modifications (*3*). The protein was analyzed by SDS−PAGE on 10−20% gradient polyacrylamide gels and stained with Coomassie blue R-250.

Preparation of PLP-Free 5,6-LAM. In the preparation of 5,6-LAM for use in PLP labeling, two methods were applied to the preparation PLP-free 5,6-LAM. In one method PLP was omitted to the purification buffer (20 mM triethanolamine hydrochloride, pH 7.2, 1 mM β-ME) to minimize adventitiously bound PLP. Alternatively, dialysis of the protein removed most of the bound PLP (*3*). Typically a small residue (less than 2%) of PLP remained bound to the enzyme.

Enzyme Activity Assay. 5,6-LAM was assayed as described (*3*), except that the preincubation of 5,6-LAM with AdoCbl was not required for the maximum activity (data not shown). The specific activity of overexpressed *P. gingivalis* 5,6-LAM was approximately 9−10 units/mg of protein, which is similar to the activity of overexpressed *C. sticklandii* 5,6-LAM (*4*). One unit of enzyme activity was defined as the amount of enzyme giving rise to 1 μmol of 2,5-DAH per minute under the standard assay conditions (*3*, *4*). In $NaBH_4$ inhibition assays, PLP-freed 5,6-LAM was combined with 1 equiv of PLP and incubated in 100 mM Epps buffer, pH 8.5, and 5 mM β-ME at 37 °C for 30 min. $NaBH_4$ was added to the activated enzyme at the end of incubation at a molar ratio of $NaBH_4$:enzyme = 33:1 before adding AdoCbl and excess PLP to start the catalytic reaction.

Enzyme Reduction by Sodium Borohydride. Stock 1 M $NaBH_4$ in 100 mM Epps buffer, pH 8.5, was freshly prepared before use. The 356 μL of PLP-saturated 5,6-LAM (303 μM) in 100 mM Epps buffer at pH 8.5 with 5 mM β-ME was reduced by 3.6 μL of 1 M $NaBH_4$. The final enzyme concentration was 300 μM, and the $NaBH_4$ concentration was 10 mM. In the radioactive labeling experiments, PLP-free 5,6-LAM (360 μM) was incubated with [U-^{14}C]PLP (360 μM), 100 mM Epps buffer (pH 8.5), and 5 mM β-ME at 37 °C for 30 min followed by reduction with $NaBH_4$. The reduced protein was separated from borohydride and residual unbound PNP on Sephadex G-25 fine, equilibrated with water. The protein fraction was carboxymethylated using the method of Imoto and Yamada (*13*). At the end of the alkylation reaction, excess reagents were removed by gel filtration through a Sephadex G-25 column (15 × 80 mm) equilibrated with 25 mM Tris-HCl, pH 8.5, and the protein was concentrated using an ultrafree-4 centrifugal filter unit (Millipore).

Endoproteinase Lys-C (Lys-C) Digestion. The concentrated protein (500 μg) for Lys-C digestion was incubated in 25 mM Tris-HCl, pH 8.5, and 1 mM EDTA with 5 μg of Lys-C at 37 °C for 18 h. The ratio of Lys-C:5,6-LAM was 1:100 (w/w) in the reaction mixture. The digestions were complete as assessed by SDS−PAGE analysis on 10−20% gradient polyacrylamide gels. Peptides (less than 10 kDa) from Lys-C proteolysis were separated from Lys-C and large peptides (>10 kDa) by a Micro-50 concentrator and submitted to peptide mapping and sequencing with Micromass Q-TOF2.

Spectrometry. UV/visible spectra were obtained on a model 8452A diode array spectrophotometer (Hewlett-Packard). Mass spectra and peptide sequencing (MS/MS) were obtained on a Micromass Q-TOF2 hydrid quadrupole/orthogonal time of light mass spectrometer at the University of Wisconsin Biotechnology Center (Madison, WI). LC/MS was performed on the same mass spectrometer equipped with an Agilent Technologies Series 1100 HPLC unit with a Vydac reverse-phase C_4 column with the detection wavelength at 214 nm. Q-TOF2 is an elctrospray LC/MS/MS tandem mass spectrometer, which is a combination of a quadrupole mass filter [MS 1], a hexapole collision cell, and an orthogonal time of flight mass analyzer [MS 2] to deliver high-performance MS/MS. The Micromass Q-TOF2 is a tandem mass spectrometer (MS/MS) with two analyzers, the first being a quadrupole analyzer that is used as an ion guide in MS mode but as a resolving analyzer in MS-MS mode. The second analyzer is a time of flight analyzer placed orthogonally to the quadrupole. The final detector is a microchannel plate detector for high sensitivity. In MS-MS mode, the two analyzers are used together for peptide sequencing by monitoring fragmentation patterns in molecules.

Peptide Mapping by LC/MS. Peptides from endoproteinase Lys-C digestion were separated on a Vydac C4 column (1 mm × 150 mm) in a gradient of 0−45% acetonitrile (v/v) in 0.1% formic acid at a flow rate of 0.2 μL/min over the course of 100 min and ramped to 81% acetonitrile in 0.1% formic acid for 5 min to elute all of the remaining peptides. A UV cell was placed in-line before the ESI source, and elution was monitored at 214 nm. All the elution buffers were filtered and degassed.

LC/MS and LC/MS/MS. The instrument operates in survey scan mode in MS/MS. Peptides are eluted at a flow rate of 0.2 μL/min and scanned as they enter the source. A MS mode scan is preferred with the collision energy off and with the quadrupole passing all ions (in RF mode). The computer scans the eluted peptides to find potential parent ions. Peptides above the threshold are subjected to MS/MS mode. When a peptide is selected, the Q-TOF is programmed to switch to MS/MS mode, which means that the computer switches the mode of quadrupole (in RFDC mode) to pass only the selected ion and with the collision energy on. Because of the use of charge recognition, the collision energy depends on the charge (z) of each peptide as well as the mass. The higher the mass (m) or the lower the charge (z) of a peptide, the higher the collision energy applied. A collision gas [argon (Ar)] is introduced into the hexapole collision cell, where the selected ion of interest (MS) collides with the argon atoms, resulting in fragmentation, and the resulting ions are scanned for several seconds. When the programmed time for MS/MS is done, the Q-TOF switches back to MS mode and resumes scanning the eluting peptides. The resulting MS/MS spectrum shows a series of predominant singly charged Y_{max} (or B_{max}) ions and C-terminal (or N-terminal) fragments of the peptide. Differences in m/z between the Y_{max} (or B_{max}) ions correspond to loss of amino acids from the N-terminus (or C-terminus), so the sequence of the original peptide can be deduced by calculating these differences.

RESULTS

Sodium Borohydride Reduction of 5,6-LAM. The prominent peak at 420 nm in the UV/visible spectrum of wild-type *P. gingivalis* 5,6-LAM is characteristic of an internal PLP-aldimine. Free PLP, on the other hand, displays a band at 385 nm (Figure 2). The 420 nm absorption band is bleached by the addition of sodium borohydride to the enzyme solution, and a new absorption band appears at 325 nm. 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 5,6-LAM activity. The activity decreased more than 40-fold when the fully saturated PLP enzyme was mixed with a 33.3-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 an essential lysine residue.

After denaturation, carboxymethylation, and proteolysis, peptides from the $NaBH_4$-reduced enzyme retained the 325 nm absorption, showing that the reduced PLP remained covalently bound to the protein (Figure 3). By contrast, the 325 nm absorption band could not be observed in the unreduced control sample, indicating the loss of PLP from the unreduced enzyme during denaturation and gel filtration in the carboxymethylation step. Moreover, the reduced protein but not the unreduced control sample contained high ^{14}C radioactivity from [U-^{14}C]PLP in our parallel radioactive labeling experiment after the same reaction (Figure 4). Taken together, the data suggested that a lysine residue intrinsic to the protein was involved in an internal aldimine linkage with PLP.

The PLP-Binding Site Resides in the Small Subunit of 5,6-LAM. A biochemical approach was adopted to identify the

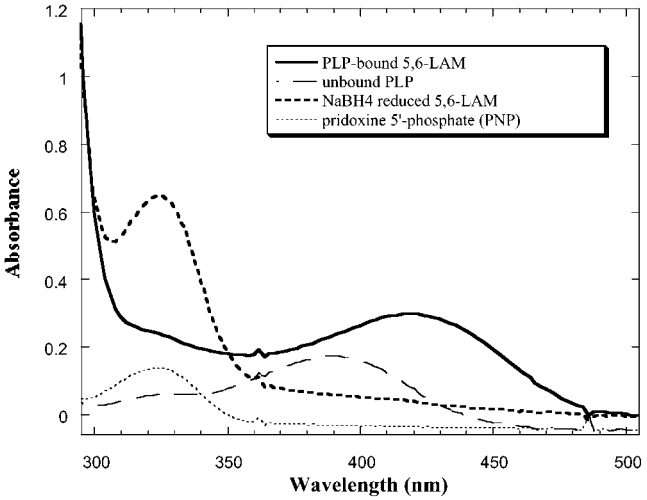

FIGURE 2: UV/visible spectral changes upon reduction of 5,6-LAM by $NaBH_4$. The internal aldimine in the active site of 5,6-LAM can be observed by its characteristic absorption at A_{420} (bold solid line), in contrast to A_{385} in the free PLP sample (solid line). Unbound PLP was excluded from all protein samples through Sephadex G-25 desalting treatment. After the saturated PLP 5,6-LAM was reduced by $NaBH_4$ (see Materials and Methods for details), the absorption peak blue shifted from A_{420} to A_{325} (solid dashed line). The A_{325} is the indication of the formation of reduced Schiff base. Pyridoxine 5′-phosphate (PNP) (dashed line) also has A_{325} absorption and is used as a control sample to indicate the formation of the reduced internal aldimine. Deionized H_2O was used as the reference.

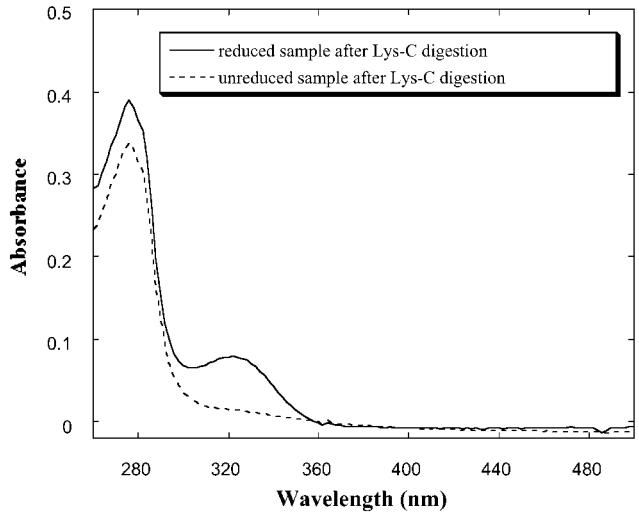

FIGURE 3: The UV/visible spectrum of $NaBH_4$-reduced and unreduced Lys-C peptides. Details of the procedures are included in Materials and Methods. The A_{325} characteristic absorption is clearly observed in the reduced peptides (solid line) but not in the control peptide sample (dashed line). Deionized H_2O was the reference.

PLP-binding site. 5,6-LAM is an $\alpha_2\beta_2$ heterotetramer with a base-off AdoCbl binding motif in the small subunit. We addressed the question of which subunit incorporates the putative PLP-binding site. To examine this, the [^{14}C]PLP-labeled 5,6-LAM was denatured and carboxymethylated, and a portion of the reduced sample was subjected to SDS−PAGE analysis. The α and β subunits were trimmed separately from the gel, and the radioactivity of each subunit was counted in a Beckman LS 6500 scintillation system. Most of the ^{14}C radioactivity was associated with the β subunit (6800 dpm/8000 dpm), where only 1000 dpm was found in the α subunit. In addition, a higher mass peak was

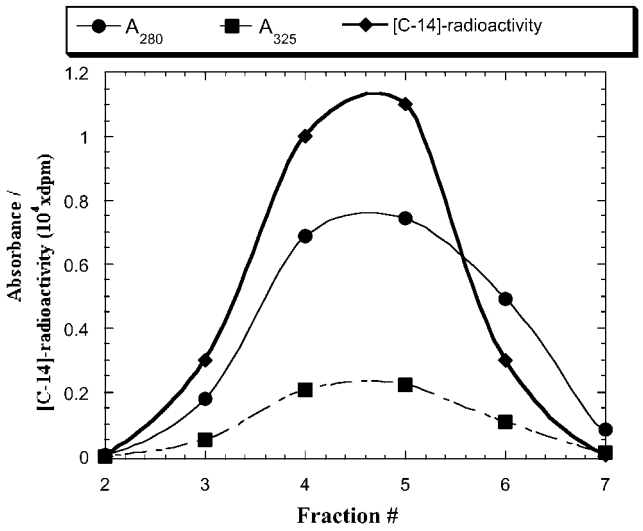

FIGURE 4: ^{14}C-Labeling of 5,6-LAM by reduction of 5,6-LAM in the presence of [^{14}C]PLP. 5,6-LAM reconstituted with [U-^{14}C]PLP was reduced with $NaBH_4$. In a parallel control experiment, the reduction with $NaBH_4$ was omitted. After denaturation and carboxymethylation, the sample was subjected to Sephadex G-25 gel filtration, with the results shown in the figure. No radioactivity was detected in the effluent in the control experiment (data not shown). The A_{325} absorption peak corresponding to the ^{14}C-labeled reduced PLP complex was observed coincident with radioactivity in the reduced sample. In the control experiment, no absorbance at 325 nm was observed (data not shown).

observed in the small subunit of the reduced protein in MALDI-TOF spectra (data not shown), which is consistent with the covalent linkage of a reduced internal aldimine. Taken together, our data indicate that PLP is covalently bound through a reduced aldimine to the β subunit of 5,6-LAM.

Identification of Peptides with the PLP-Binding Site. To identify the active site lysine residue, a few methods for degradation to peptides were evaluated. Endoproteinase Lys-C proved to fit our requirements and provided satisfactory results. Lys-C peptides from the $NaBH_4$-reduced and the unreduced protein were separated by HPLC (part of LC/MS) on a C4 reverse-phase column (Vydac), and the chromatograms were compared (Figure 5). In the HPLC of peptides of Lys-C digestion from the $NaBH_4$-reduced protein (Figure 5B), a fraction emerged at 66 min that was absent in the HPLC of peptides from the unreduced protein (Figure 5A). Moreover, two fractions in the control sample, retention times 49 and 63 min, were decreased in the reduced sample.

To identify the essential lysine residue, sequence information from each peak was obtained on a Micromass Q-TOF2. After analysis by this method, we could unambiguously identify most of the peptides from the Lys-C proteolysis of 5,6-LAM in both the $NaBH_4$-reduced and unreduced samples, and most of the identified peptides could be sequenced (Table 1). Two mass peaks, which were strong in the unreduced sample but not in the reduced sample that appeared in the 49 min fraction, were found at masses 2410.2 and 2516.2, and the peaks were derived from peptide β121−β144. The mass of peptide β121−β144 should be 2400.2, the difference being due to the carboxymethylation ($m + 58$) (*17*−*21*) of two methionine residues of the peptide, with decomposition of one *S*-carboxymethylmethionine sulfonium ion under the acidic conditions (*17*). Furthermore, two strong peaks in the

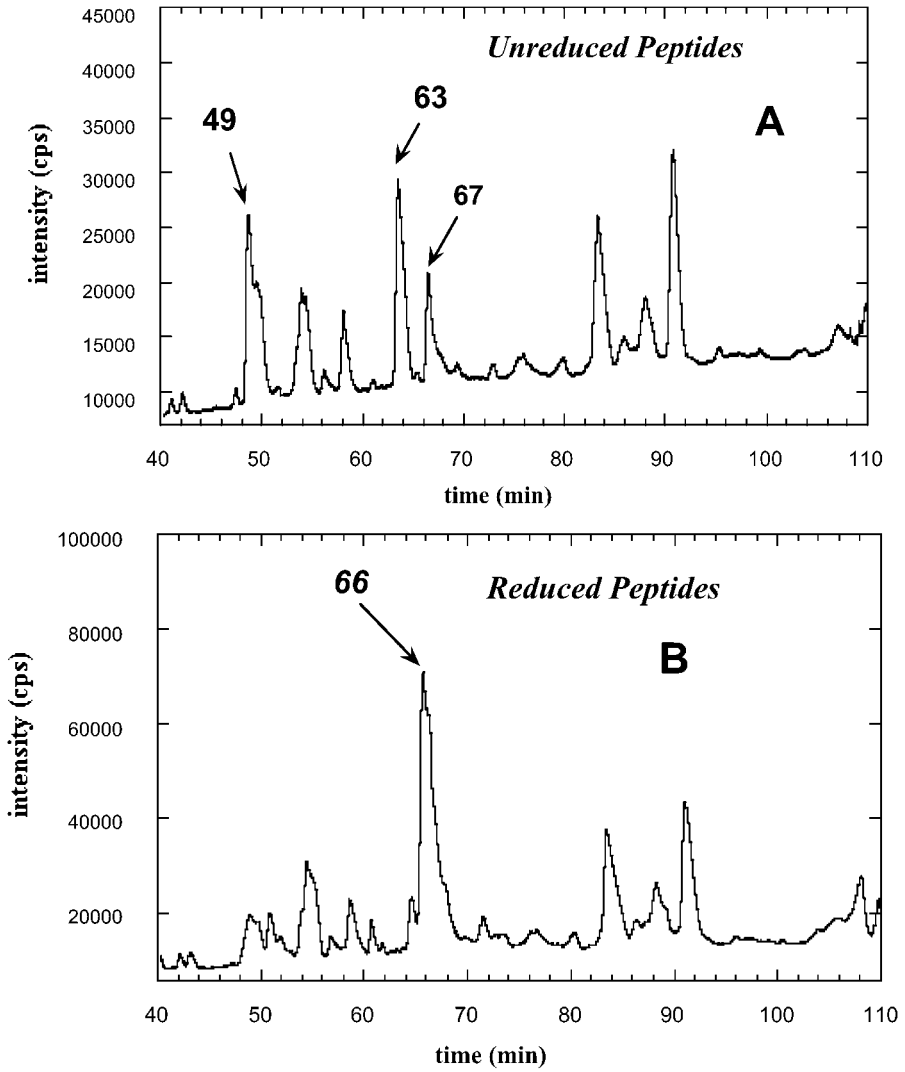

FIGURE 5: Peptide mapping of the unique PLP-modified peptide. In both chromatograms, fractions were monitored at 214 nm. The reduced and control peptides were subjected to Micromass Q-TOF2 to identify the peptide containing the PLP-labeled lysine (see Materials and Methods for details). In the peptide mapping of reduced peptides (panel B), the intensities of two major peptide peaks in the unreduced sample (panel A), at 49 and 63 min (marked by an arrow), are diminished. A unique and strong new peak emerged at ~66 min (marked by an arrow).

63 min fraction of the unreduced sample were observed at masses 3590.7 and 3484.7, and the corresponding peptide was β145−β175. A methionine residue in the peptide β145−β175 was carboxymethylated (3590.7) and partially decomposed (3484.7). In contrast, the MS/MS sequencing was complete whenever a peptide was free of methionine (Table 1).

In contrast, in the $NaBH_4$-reduced sample the foregoing peptides were absent, and a new unique and strong peak of mass 6318.8 Da appeared in the 66 min fraction. The MS/MS data of this fraction give the sequences of 10−15 amino acids from the N- and C-termini of the peptide. The N-terminal peptide sequence IVVVVGASTGTDAHTVGI is identical to the first 18 amino acids of peptide β121−β144 identified above, and the C-terminal sequence KAIFEENPVQSG is identical to the C-terminal 12 amino acids of peptide β145−β175 also identified above. The full sequence could not be obtained owing to the mass of the peptide and the complication of methionine residues. However, the available sequence information identified the 55-residue peptide (6318.8 Da) as derived from β121 to β175 (6088 Da) of *P. gingivalis* 5,6-LAM, plus 231.1 mass units due to the linkage of the PLP moiety.

Furthermore, in separate experiments we studied the Lys-C peptides derived from protein alkylated under conditions of decreased alkylation of methionine (see below). Under these conditions the masses of major peptides in the 49 and 63 min fractions were found to be 2400.2 and 3532.7 Da, respectively, corresponding exactly to the calculated masses of peptides β121−β144 (2400.2 Da) and β145−β175 (3532.7 Da). Moreover, in Lys-C proteolysis of a $NaBH_4$-reduced sample with decreased alkylation of methionine, the mass of the 66 min fraction was 6145 Da, consistent with the predicted mass for peptide β121−β175 plus 231 for the linked PLP linkage. Taken together, the data suggested that K144(β) was covalently modified by PLP, imparting resistance to Lys-C cleavage at this position. Furthermore, our data also provide an explanation for why endoproteinase Lys-C failed to cleave at K144(β) in the $NaBH_4$-reduced sample but not the control.

Examining the amino acid sequences of 5,6-LAM from *C. sticklandii* and *P. gingivalis*, as well as that of D-ornithine

Table 1: Characterization of Peptide Fractions from Q-TOF/LC/MS and Q-TOF/LC/MS/MS

peak	t_R[a]	m	assigned peptides	corresponding sequence[d,e]	footnote
1	41	855	α(431−437)[c]	**YIFS**NM*K-106	
		960	α(431−437)[b]	**YIFS**NM*K	
2	42	735.4	α(481−487)	**GIFGDVK**	
		765.4	α(6−12)	**VGIDFSK**	
		1022.5	α(438−446)	**EEGLFSALEK**	
		1656.9	α(447−461)	**EGGIVQSRAREVLDK**	
3	48−49	1602.8	α(503−515)[c]	**ELHYYNPTIE**LM*K-106	
		1708.8	α(503−515)[b]	**ELHYYNPTIE**LM*K	
		2410.2	β(121−144)[c]	**IVVVGASTGTDAHTVGI**DAIM*NM*K-106	*f, j*
		2516.2	β(121−144)[b]	**IVVVGASTGTDAHTVGI**DAIM*NM*K	*f, j*
4	54	1745.9	β(216−231)[c]	M*IL**ACGGPRITYELAK**-106	
		1851.9	β(216−231)[b]	M*IL**ACGGPRITYELAK**	
		2048.0	β(104−120)[c]	WDM***AETDDFIRENIGRK**−106	
		2154.0	β(104−120)[b]	WDM***AETDDFIRENIGRK**	
5	58	1122.6	α(471−480)	**EEGLFSALEK**	
		2689.2	β(1−23)	VM**SGGLYSTEGRDFDQTLDLSRIK**	*h*
6	63	1900.0	β(176−193)	**GIELNADALLVSQTVTQK**	
6	63	3484.7	β(145−175)[c]	GFAGHYGLERYEM*IEAYNLGSQVPNEEFIAK-106	*f, i*
		3590.7	β(145−175)[b]	GFAGHYGLERYEM*IEAYN**LGSQVPNEEFIAK**	*f*
7	66−67	1924.0	β(199−215)[c]	**NLIELV**ELM*E**AEGLRDK**-106	
		2030.0	β(199−215)[b]	**NLIELV**ELM*E**AEGLRDK**	
		2056.1	β(34−53)	**VQLSFTLPVPAGDEAIEAAK**	
		2271.2	α(69−90)	**DRNVLGHGALFFLANGIVATGK**	
		6318.8	PLP plus β(121−175)[b]	**IVVVGASTGTDAHTVG**IDAIM*NM*KGFAGHYGL-ERYEM*IEAYNL**GSQVPNEEFIAK**	*g, j*
8	83	2936.3	β(232−258)	**ELGYDAGFGANTYADDVASFIAQEFHK**	
9	88	4903.1	β(59−98)[b]	M*GM***QNPQVVFFRELTEGFTFFNCYGSCEHTVDYSSIYVPK**	
10	91	4338.1	α(91−131)[b]	**SPQEIAESVAVGEVDLTSYPFCSANVIADTLAPLI**TGGM*QK	
		4280.2	α(91−131)	**SPQEIAESVAVGEVDLTSYPFCSANVIADTLAPLIT**GGMQK	*h*
		6154	α(378−430)[b]		*i*
11	109	6081.1	α(13−68)		*i*
		16962, 17020	α(220−371)[b]		*i*

[a] Retention time. [b] Methionine residues (M) are modified by carboxymethylation [m/z + (58 × number of Met)]. [c] The decomposition of the *S*-carboxymethylmethionine sulfonium occurs [m/z + (58 × number of Met) − 106]. [d] All of the cysteine residues were carboxymethylated, and methionine residues are either modified by carboxymethylation or degraded. [e] Sequenced residues are in boldface; underlined residues are the conserved AdoCbl binding motif. [f] Strong in the controlled sample. [g] Found only in the $NaBH_4$-reduced sample. [h] No methionine modified. [i] No sequence information. [j] K stands for the PLP-binding lysine.

aminomutase from *C. sticklandii* and *C. difficile*, the active site lysine identified in this paper is conserved in all four sequences (Figure 7). The PLP-binding site is fairly close, less than 10 amino acid residues separated from the highly conserved base-off AdoCbl binding site (DXHXXG).

N-Terminal Valine in the Small Subunit. The amino acid sequence of the β subunit as translated from the gene would begin with methionine. However, as shown in Table 1, the N-terminal peptide β(1−23) displays a mass of 2689.2, and the sequence begins with valine at position 1 followed by methionine in position 2. The codon of the gene corresponds to methionine 2. In this case, the translated amino acid sequence differs from the actual sequence by the presence of valine 1 in the purified protein, and translation is initiated one codon ahead of the putative start codon.

Site-Directed Mutagenesis of Lysine 144 in the β Subunit. To determine whether an internal aldimine between PLP and lysine 144(β) is absolutely required for catalysis, site-directed mutagenesis was performed to replace lysine 144 with a glutamine residue. In the purification of K144Q(β)-5,6-LAM, normal elution behavior was observed both in DE52 ion-exchange chromatography and in phenyl-Sepharose 6 Fast Flow hydrophobic chromatography. After passage of K144Q-(β)-5,6-LAM though a short G-25 column to remove most of the free PLP, which had been included in the purification buffer, the protein appeared colorless. In contrast, all of the other mutated and wild-type samples of 5,6-LAM displayed the yellow color of the PLP-aldimine. The characteristic absorption band at 420 nm of an internal aldimine was absent in the visible spectrum of K144Q(β)-5,6-LAM (Figure 6).

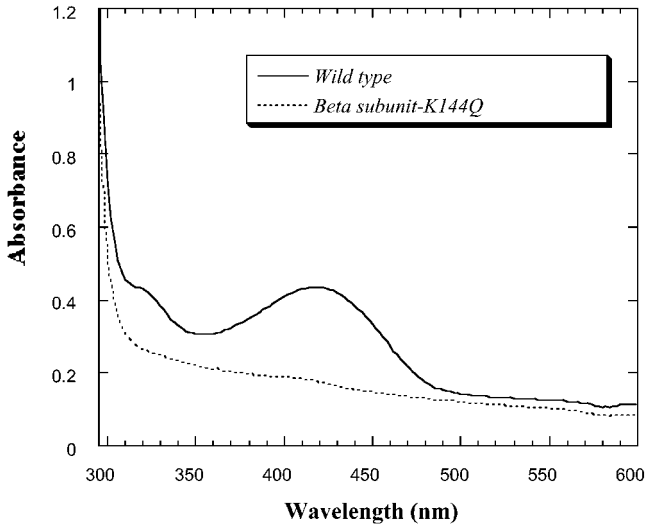

FIGURE 6: UV/visible spectra of purified wild-type 5,6-LAM and K144Q(β)-5,6-LAM. Wild-type 5,6-LAM and the mutated variant of 5,6-LAM were purified in the presence of PLP in the purification buffer, and free PLP was removed from the protein sample by Sephadex G-25 chromatography. The band at 420 nm characteristic of the internal PLP-aldimine in the wild-type enzyme is not observed in the K144Q(β)-5,6-LAM.

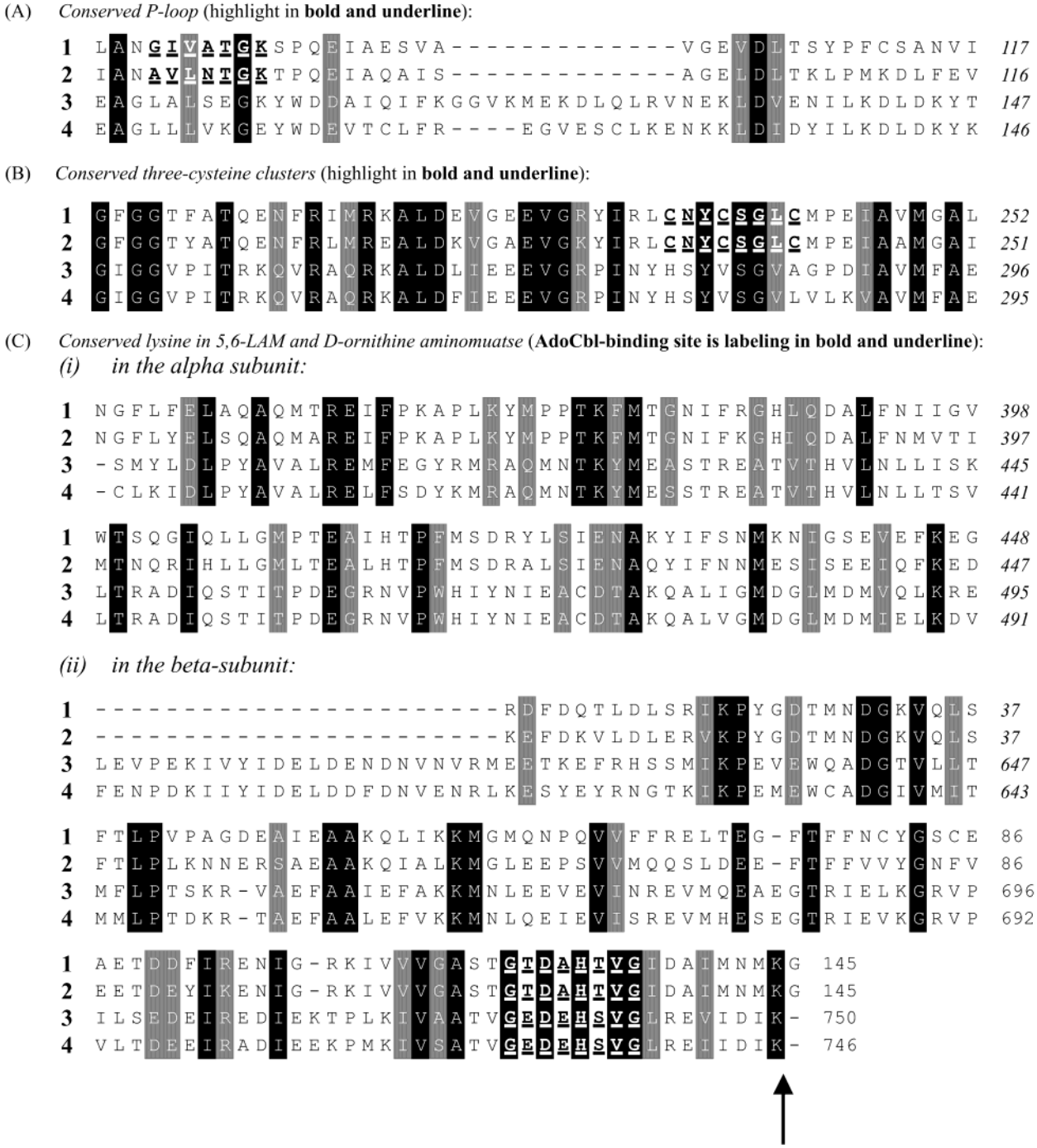

FIGURE 7: Partial sequence alignments of 5,6-LAM and related enzymes. Amino acid sequences of 5,6-LAM from *P. gingivalis* (**1**) and *C. sticklandii* (**2**) are aligned with the corresponding sequences of D-ornithine aminomutase from *C. sticklandii* (**3**) and *C. difficile* (**4**), respectively. Identical residues in the alignments are highlighted in black background, and similar residues are shown in gray background. The PLP-binding lysine 144β in 5,6-LAM is indicated by an arrow.

Furthermore, in the activity assay of K144Q(β)-5,6-LAM, formation of 2,5-DAH could not be detected even in assays with prolonged incubation. In summary, the absence of internal aldimine and the catalytic activity in K144Q(β)-5,6-LAM indicate that lysine 144 in the β subunit is a functionally important residue and that PLP binds to the active site by forming an internal aldimine with the ϵ-amino group of this lysine residue.

Site-Directed Mutagenesis of Other Residues. From the sequence alignment of two different species of 5,6-LAM, a consensus P-loop sequence, A(G)XXXXGKT(S) (84α−90α), was identified (Figure 7). This hypothetical P-loop was investigated by site-directed mutagenesis of lysine 90(α) to glutamine in the *P. gingivalis* 5,6-LAM. The catalytic activity was evaluated, and UV/visible spectra were obtained. Table 2 shows the comparisons of enzymatic activity of *P. gingivalis* K90Q(α)-5,6-LAM with that of the wild-type enzyme. The K90Q(α)-5,6-LAM activity was as high as 60% of that of the wild-type enzyme. Therefore, the P-loop function is unlikely to be involved in catalysis.

Another conserved motif appearing in 5,6-LAM is a three-cysteine motif (CNYCSGLC, 235α−242α) (Figure 7). From previous studies (*4*), the sequence appeared similar to the structural zinc site in alcohol dehydrogenase, but no signifi-

Table 2: Specific Activities of the Wild-Type and Mutated Lysine 5,6-Aminomutases from *P. gingivalis*

lysine 5,6-aminomutase	specific activity[a] (units/mg)
wild type	8.31 ± 0.78
K90Q(α) (P-loop mutant)	6.02 ± 0.96
C235S(α)-C238S(α)-C242S(α) (three-cysteine cluster) mutant	0.75 ± 0.13
K377Q(α)	0.05 ± 0.005
K446Q(α)	2.13 ± 0.09
K23Q(β)	6.20 ± 0.44
K58Q(β)	2.75 ± 0.13
K144Q(β)	0

[a] Activity assays were carried out under standard conditions as described (*3*, *4*).

cant loss in catalytic activity was found by removing zinc from 5,6-LAM. In mutagenesis experiments, we replaced the three cysteine residues with serine. About 10% activity remained in the triply mutated C235S(α)-C238S(α)-C242S(α)-5,6-LAM. This activity suggested that the three-cysteine cluster is not critical for catalysis.

To understand whether other conserved lysine residues from the sequence alignment of 5,6-LAM and D-ornithine aminomutase (Figure 7) were critical for catalytic activity, two conserved lysine residues in the α subunit, K377(α) and K446(α) of 5,6-LAM, were also investigated by mutation to glutamine residues. These two lysine residues had been proposed to be PLP-binding sites (*9*). D-Ornithine aminomutase is another example of PLP- and a base-off-type AdoCbl-dependent aminomutase catalyzing the reversible interconversion of D-ornithine to (2*R*,4*S*)-2,4-diaminopentanoic acid as the second step in the oxidative pathway of L-ornithine in *C. sticklandi*i (*1*, *9*). In our activity study, K377(α) is obviously more important for the catalysis in 5,6-LAM than K446(α) (Table 2). However, both displayed significant activities, in contrast to K144Q(β)-5,6-LAM.

DISCUSSION

An Essential Lysine in a Novel 5,6-LAM PLP-Binding Site. In the proposed mechanism of action of 5,6-LAM, substrate- and product-based radical intermediates are linked to PLP through the formation of external aldimine adducts. In search of an internal PLP-aldimine at the active site, we have clearly demonstrated the existence of an essential lysine, K144(β), that is critical for the catalytic activity of 5,6-LAM through the formation of an internal aldimine linkage. The PLP-binding site is novel (*14*−*16*) and different from that of the previously proposed lysine residues (*9*). The binding site of PLP is highly conserved between 5,6-LAM from *C. sticklandii* and *P. gingivalis*, so it is more than likely that the same lysine is the PLP-binding site in *C. sticklandii* 5,6-LAM (Figure 7C). This conserved lysine also appears in the D-ornithine aminomutase from *C. sticklandii* and *C. difficile*, which also has a conserved AdoCbl binding motif nearby.

The PLP-binding sequence in 5,6-LAM and D-ornithine aminomutase differs from those in other PLP-dependent enzymes. Typical PLP-binding motifs found in other PLP enzymes, such as SXXK(PLP) (*14*, *15*) or the motif PGGGGK(PLP) in LAM (*16*), do not appear in 5,6-LAM from *C. sticklandii* or *P. gingivalis*.

Only Conserved Lysine 144(β) Is Critical for the Activity of 5,6-LAM. From the amino acid sequence alignment of 5,6-LAM and D-ornithine aminomutase, five lysine residues are conserved among them (Figure 7C). On the basis of the similarity between these two AdoCbl-dependent enzymes (*9*), one of the lysine residues should be responsible for binding PLP through an internal aldimine. In the mutagenesis study (Table 2), only K144Q(β)-5,6-LAM lacked observable activity. Each of the other four conserved lysine residues was replaced with a glutamine residue, and all were catalytically active. Moreover, in peptide mapping studies, peptides containing each conserved lysine were characterized and sequenced by Q-TOF/MS or Q-TOF/MS/MS as demonstrated in Table 1. Taken together, our data indicated that only the conserved lysine 144(β) is essential for 5,6-LAM activity.

Alkylation of Methionine Residues in 5,6-LAM by Iodoacetate. As we described before, the percentage of methionine residues in 5,6-LAM is much higher than normal proteins. In addition, most of the methionine residues were modified by iodoacetic acid during the carboxymethylation reaction. The overmodification of methionine residues in 5,6-LAM is very unique. Although the carboxymethylation of methionine has been reported in the literature (*17*−*21*), it is uncommon to observe a large percentage of methionine residues in the protein modified. We did not observe modification of methionine residues in the small protein Fhit (fragile histidine triad) in Q-TOF/MS when it was treated by the same procedure as described in Materials and Methods. Fhit contains only three methionine residues. We duplicated the carboxymethylation reaction of 5,6-LAM by exposure to different denaturing agents, 6 M Gdn-HCl or 8 M urea, and the modification of methionine residues was reproducible. Substantially more modification of methionine residues occurred in 6 M Gdn-HCl than in 8 M urea, although about 25% of the methionine residues were alkylated in 8 M urea. This suggested that the carboxymethylation of methionine residues is accelerated by the high ionic strength associated with 6 M Gdn-HCl. The fact that over-carboxymethylation of methionine in 5,6-LAM is observed in 8 M urea as well as in 6 M Gdn-HCl is attributed to the high content of methionine in the protein (31 residues). This amplifies any lower background level of modification that could be overlooked in proteins with 5−10 methionine residues.

A PLP-binding site has never before been identified in an AdoCbl-dependent enzyme. The present study represents the first report of a novel PLP-binding site in an AdoCbl-dependent enzyme, and the PLP-binding site is very close to a conserved AdoCbl binding motif. It could shed light on the identification of the PLP site of AdoCbl- and PLP-dependent enzymes, such as D-ornithine aminomutase and other unknown novel aminomutases in amino acid metabolism. Future work in this laboratory will focus on the determination of the role of reactivation of E_2 protein through the overexpression of E_2 protein from *P. gingivalis* and the identification of a mechanism-based organic radical.

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