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Identification of Structural and Catalytic Classes of Highly Conserved Amino Acid Residues in Lysine 2,3-Aminomutase †

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Abstract

Lysine 2,3-aminomutase (LAM) from *Clostridium subterminale* SB4 catalyzes the interconversion of (S)-lysine and (S)-β-lysine by a radical mechanism involving coenzymatic actions of S-adenosylmethionine (SAM), a [4Fe-4S] cluster, and pyridoxal-5'-phosphate (PLP). The enzyme contains a number of conserved acidic residues and a cysteine and arginine-rich motif, that binds iron and sulfide in the [4Fe-4S] cluster. The results of activity and iron, sulfide, and PLP analysis of variants resulting from site-specific mutations of the conserved acidic residues and the arginine residues in the iron-sulfide binding motif indicate two classes of conserved residues of each type. Mutation of the conserved residues Arg134, Asp293, and Asp330 abolish all enzymatic activity. Based on the x-ray crystal structure, these residues bind the ε-aminium and α-carboxylate groups of (S)-lysine. However, among these residues only Asp293 appears to be important for stabilizing the [4Fe-4S] cluster. Members of a second group of conserved residues appear to stabilize the structure of LAM. Mutations of arginine residues 130, 135, and 136 and acidic residues Glu86, Asp165, Glu236, and Asp172 dramatically decrease iron and sulfide contents in the purified variants. Mutation of Asp96 significantly decreases iron and sulfide content. Variants in Arg130 or Asp172 display no detectable activity, whereas variants in the other positions display low to very low activities. Structural roles are assigned to this latter class of conserved amino acids. In particular, a network of hydrogen bonded interactions of Arg130, Glu86, Arg135 and the main chain carbonyl groups of Cys132 and Leu55 appears to stabilize the [4Fe-4S] cluster.

Lysine 2,3-aminomutase (LAM)¹ catalyzes the interconversion of (S)-lysine and (S)-β-lysine, a reaction that proceeds by intramolecular migration of the α-amino group to the β-carbon with inversion of configuration and concomitant transfer of the 3-pro-R hydrogen in (S)-lysine to the 2-pro-R position of (S)-β-lysine (1,2). The enzyme contains a [4Fe-4S] cluster (3) and PLP and is activated by SAM. Hydrogen transfer is mediated by C-5' of the adenosyl moiety in SAM by a radical mechanism (4–7). Reversible reductive cleavage of SAM by the [4Fe-4S] cluster according to eq. 1 generates the 5'-deoxyadenosyl radical (8,9), which in turn initiates the rearrangement reaction by the mechanism shown in Fig. 1. Abstraction of the 3-pro-R hydrogen from the lysyl side chain of the PLP-lysyl external aldimine by the 5'-deoxyadenosyl

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¹Abbreviations: LAM, lysine 2,3-aminomutase; SAM, S-adenosyl-L-methionine; SeSAM, Se-adenosyl-L-selenomethionine; PLP, pyridoxal-5'-phosphate; IPTG, isopropylthiogalactoside; DTT, dithiothreitol; PITC, phenylisothiocyanate; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; PCR, polymerase chain reaction; WT, wild type

radical forms 5'-deoxyadenosine as a tightly bound intermediate. The resulting PLP-lysyl radical **1** undergoes isomerization by way of the azacyclopropylcarbinyl radical **2** to the product-related PLP-β-lysyl radical **3**. Abstraction of a hydrogen atom from the methyl group of 5'-deoxyadenosine by radical **3** forms the PLP-β-lysyl external aldimine and the 5'-deoxyadenosyl radical, completing the isomerization cycle (10,11).

PLP is bound to LAM as an internal aldimine with Lys337 (12,13). Reaction with (*S*)-lysine by transaldimination frees Lys337 and generates the external PLP-lysyl aldimine (Fig. 1). A structure of the external aldimine shows the ε-aminium group of the lysyl side chain in hydrogen bonded contacts with Asp293 and Asp330 (13). Asp293 is highly conserved among species of LAM, and Asp330 is largely conserved but appears to be glutamate in a few species. The specificity of LAM for (*S*)-lysine may be determined by the interactions of Asp293 and Asp330 with the lysyl side chain.

LAM is a member of the radical SAM superfamily of enzymes that incorporate the generic sequence CxxxCxxC as the basic binding motif for iron and sulfide in [4Fe-4S] centers (14). In LAM, the amino acid sequence of the iron and sulfide binding site is ₁₂₅CSMYCRHCTRRR₁₃₆; the motif includes the absolutely conserved Arg130 and is flanked by three downstream arginine residues, two of which are conserved in homologous proteins found in the bacterial genome databases (15). The significance of these arginine residues is of interest. In this paper, we report the properties of specifically mutated variants of LAM, in which conserved arginine residues in the iron-sulfur motif are mutated, and of variants in which conserved acidic amino acids are mutated. The results are correlated with the structure of LAM in complex with (*S*)-α-lysine, PLP, and SeSAM (13).

MATERIALS AND METHODS

Coenzymes and Chemicals

Difco Terrific Broth, (*S*)-lysine monohydrochloride, FeSO₄, ampicillin, and tetracycline were obtained from Fisher; SAM, PLP, β-mercaptoethanol, DTT, streptomycin sulfate from Sigma; standard iron solution from Aldrich; PITC from Pierce Chemical Co.; imidazole Ultrol Grade from Calbiochem; Sephacryl S-200 HR, phenyl-Sepharose 6 Fast Flow High Substitution, and Q-Sepharose Fast Flow from Amersham Biosciences; pET23a(+) vector, Ni-NTA His Bind Resin - Novagen; Pfu DNA polymerase, QuikChange Site-Directed Mutagenesis Kit, *E. coli* Epicurian *coli* XL2-Blue MRF⁺ supercompetent cells - Stratagene; *Nde*I and *Xho*I restriction endonucleases - Promega; Zero Blunt TOPO PCR Cloning Kit - Invitrogen; Centricon YM10 - Millipore; and (*S*)-[¹⁴C]lysine from NEN Life Sciences. SAM was purified by chromatography through a 2 × 20 cm column of CM-cellulose eluted with 20 mM H₂SO₄ and was stored at -70 °C. Oligonucleotide primers for cloning and site-directed mutagenesis were obtained from the DNA Synthesis and Sequencing Laboratory, Biotechnology Center, University of Wisconsin-Madison.

Expression of LAM

The original expression system has *kamA* from *C. subterminale* inserted into pET23a(+) to give pAF-8/*kamA*. The latter is transformed into *E. coli* BL21(DE3) cells containing *palter-Ex2/argU*, expressing tRNA for rare codons AGA and AGG (15). Expression of *kamA* is enhanced by co-expression of *argU* (15). To facilitate purification, a vector expressing His-

tag LAM (C-terminus) was prepared from the original construct by removing the stop codon. Included in pET23a(+) is a series of six codons encoding hexahistidine after the multiple cloning site. Removing the stop codon for *kamA* allows expression of the hexahistidine tag. The pUC19 vector, E138 (15) containing the entire gene *kamA* from the genomic library was used as template. The following PCR primers for pET23a(+) including *NdeI* and *XhoI* restriction sites and excluding the stop codon were used: plus strand, 5'-TACACATATGATAAATAGAAGATATG, and minus strand, 5'-TAGACTCGAGTTCTTGAACGTGTCTC. PCR was conducted using Pfu DNA polymerase. The resulting blunt-ended oligonucleotide was ligated to pCR BluntII TOPO vector. Digestion with *NdeI* and *XhoI* and purification by 2% agarose electrophoresis resulted in a 1.2 kb fragment ligated to pET23a(+) similarly cut with *NdeI* and *XhoI* and dephosphorylated. After transformation into *E. coli* XL2-Blue MRF' competent cells, purified plasmid DNA was sequenced to verify the construct and used to transform *E. coli* BL21(DE3)/pAlter-Ex2 *argU* cells.

Site Directed Mutagenesis

Site directed mutagenesis was conducted using the PCR-based QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primers used for plus strands are listed in Table 1, and complementary primers were the (−) strands. For PCR, 5–10 ng of template [pET23a(+)/*kamA* WT or pET23a(+)/*kamA* WT His-tag] and 125 ng of each primer were used. Mutated vectors were initially transformed into *E. coli* XL1 Blue competent cells. Plasmids obtained from colonies were sequenced in entirety and used to transform *E. coli* BL21(DE3)/pAlter-Ex2/*argU* cells.

Expression of Mutated LAM

Cells were cultured in Terrific broth containing 100 µg/mL ampicillin and 10 µg/mL tetracycline and supplemented with 50 µM FeCl₃, 50 µM ZnSO₄, and 1 mM IPTG. Two-liter shake flasks containing 1.5 L of media were inoculated with a 6 h culture of each *E. coli* cell line. Cells were cultured for approximately 24–30 h at 37 °C with slow shaking (50–75 rpm) to minimize exposure to oxygen. Following cell culture, cells were harvested by centrifugation; cell pellets were frozen in liquid nitrogen and stored at −70 °C.

Purification of Wild-type and Mutated LAM

All purification procedures were performed inside an anaerobic chamber (Coy, Grass Lake, MI). Wild type LAM and R130Q-, R134Q-, R135Q-, and R136Q-LAM were expressed and purified without His-tags as described (15). Protein yields from 30 g cells were: wild type, 500 mg; R130Q-, 25 mg; R134Q-, 375 mg; R135Q-, 45 mg; and R136Q-LAM, 100 mg.

His-tagged wild type and mutated LAM were purified in the anaerobic chamber as follows. Approximately 30–40 g of frozen cells were added to 200 mL of lysis buffer (consisting of 50 mM KPi at pH 8.0, 300 mM KCl, 0.1 mM (S)-lysine, 10 µM PLP, 2 mM β-mercaptoethanol, and 10 mM imidazole). Cells were sonicated with six 30 s bursts (Fisher Sonic Dismembrator Model 550) at a setting of 8, keeping the temperature between 4 and 8 °C. After sonication, cells were centrifuged at 100,000 × g for 30 min at 4 °C. Supernatant fluids were applied directly to Ni-NTA His-Bind columns (4 to 5 cm by 2 to 2.5 cm) previously equilibrated with lysis buffer. After washing with 200 mL of lysis buffer, the proteins were eluted with lysis buffer containing 175 mM imidazole. Imidazole-eluates were passed through a Sephacryl-S200 column (5 × 30 cm) equilibrated with standard buffer (SB is 30 mM K-EPPS at pH 8.0, 20 µM FeSO₄, 10 µM PLP, 0.1 mM (S)-lysine, and 1 mM EPPS). Pooled fractions were concentrated using Centricon YM10 spin columns to approx. 20–30 mg/mL prior to freezing and storage in liquid nitrogen. R136K-LAM was not produced from the mutated gene and could not be

purified. Typical His-tag protein yields from 30 g cells: wild type, 400 mg; R130K-, 150 mg; R134K-, 290 mg; R135K-LAM, 90 mg.

Assays

LAM activity was assayed inside the anaerobic chamber as described (6). LAM was analyzed for iron, inorganic sulfide, and PLP by published methods (16–18).

The concentration of purified wild type LAM was determined spectrophotometrically, using the value $\epsilon_{280} = 3.6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (19) and correlated to the BCA protein assay (Pierce Chemical). Concentrations of mutated LAM could not be accurately measured by absorbance at 280 nm using the extinction coefficient for the wild type enzyme because many mutations led to variations in content of [4Fe–4S] clusters, which contribute to the absorption at 280 nm. Instead, the concentrations of mutated LAM were measured by use of the BCA protein assay (Pierce Chemical Co.) and correlated with a standard solution of wild-type LAM, the concentration of which had been determined by amino acid analysis as follows. Samples of purified *C. subterminale* SB4 LAM and the iron-sulfur reconstituted LAM were subjected to complete hydrolysis in 6 N HCl at 110 °C. Protein (5 μ l, 100–125 μ g) was added to hydrolysis tubes (Pierce Chemical Co., Rockford, IL) containing 700 μ l 6N HCl. To half of the tubes, 25 μ l of an amino acid standard solution (2.5 mM of each amino acid – Pierce Chemical Co., Rockford, IL) was added in addition to protein. Tubes were sealed *in vacuo* and heated for 24, 48, and 72 hr at 110 °C. Following hydrolysis, samples were transferred to 2 mL microcentrifuge tubes and concentrated to dryness by vacuum centrifugation. The dried samples and amino acid standards were derivatized with PITC and separated by HPLC as described (20). Dried samples were dissolved in 100 μ l of coupling buffer, concentrated to dryness by vacuum centrifugation, redissolved in 100 μ l coupling buffer plus 10 μ l of PITC, allowed to react for 30 min at room temperature, concentrated to dryness as above, and dissolved in 1 mL water prior to injection and separation by HPLC using a Beckman Gold HPLC. The PITC derivative of each amino acid was separated by HPLC chromatography using a C18 reverse phase column (Phenomenex Gemini 5 μ , 4.6 mm $\times$ 25 cm, #G-4435-EO), flow rate 1 mL/min, room temperature, sample injection volume 50 μ l. The eluting compounds were detected by spectrophotometry at a wavelength of 254 nm. The amino acids were resolved with a complex linear gradient composed of buffer A: 0.05 M ammonium acetate in water and buffer B: 0.10 M ammonium acetate in 44% water, 46% acetonitrile, 10% methanol. The gradient established was 0% Buffer B in 5 min, 0–10% Buffer B in 40 min, 10–35% Buffer B in 35 min, 35–55% Buffer B in 35 min, 55–100% Buffer B in 10 min. The PITC derivatives of each amino acid were eluted at the following retention times in minutes: Asx, 13; Glx, 18; Ser, 36; Gly, 39; His, 54; Thr, 56; Ala, 58; Arg, 60; Pro, 62; Tyr, 84; Val, 85; Met, 89; Ile, 98; Leu, 99; Phe, 108; and Lys, 117.

Reconstitution of [4Fe–4S] clusters

Reconstitution of wild type and mutated lysine 2,3-aminomutase was conducted anaerobically by the method of Hewitson et al.(21). To 200 μ M (subunit) of enzyme was added 1.2 mM FeSO₄, 1.2 mM Na₂S and 5 mM dithiothreitol. The samples were incubated at room temperature for 4 hours prior to gel filtration with Sephacryl S200 HR column (2.5 $\times$ 25 cm) equilibrated with SB buffer to separate excess reagent from protein. Protein fractions were collected and concentrated using Centricon YM10 spin columns (Amicon) to approx. 20–30 mg/mL prior to freezing and storage in liquid nitrogen.

RESULTS AND DISCUSSION

Conserved amino acid residues in LAM

More than fifty genes in bacterial genomes have been annotated as encoding LAM. The graphic in Fig. 2 depicts the most highly conserved segments of amino acid sequences in 55 species of LAM, showing fully and highly conserved residues. Examples of fully conserved residues include the cysteine residues 125, 129, and 132 in the iron and sulfide binding motif, as well as a number of acidic and basic residues. Some of the latter are engaged in binding the substrate or PLP. For example, the ϵ -amino group of the conserved Lys337 binds PLP in the internal aldimine complex and is displaced by the α -amino group of the substrate lysine in the external aldimine complex depicted in Fig. 3 (12,13). In contrast, Fig. 2 shows that aspartate appears most frequently at position 330, as it does in *C. subterminale* LAM; however, glutamate appears at this position in *E. coli* LAM and a number of other species. In the following sections, we explore the functional roles of conserved acidic and basic amino acid residues in LAM from *C. subterminale*.

The ϵ -aminium group of lysine

The specificity of LAM for (*S*)-lysine as its substrate implies the existence of a binding locus for the ϵ -aminium group. In addition, the α -amino group engages in aldimine formation with PLP. Because the ϵ -aminium group is likely to be bound through an electrostatic interaction with an acidic residue of the enzyme, we mutated the conserved glutamate and aspartate residues and searched for a variant that lacked enzymatic activity but retained PLP, iron, and sulfide.

Purification of the wild-type and mutated LAM by the usual procedure, including hydrophobic and ion exchange chromatographic steps, routinely decreases the content of iron, sulfide, and PLP to amounts corresponding to less than one PLP and one [4Fe–4S] per subunit in the wild-type enzyme. The iron and sulfide can be restored by reconstitution, and PLP is added back in the standard assay.

In our initial experiments, however, we measured iron, sulfide, and PLP without reconstitution to determine the consequences of mutation on the overall coenzyme content. Any mutation that weakened the binding of PLP and/or the [4Fe–4S] cluster would confer low activity for that reason irrespective of the binding of the substrate. Then, any variant lacking a specific glutamate or aspartate that contained substantial amounts of coenzymes, but was entirely inactive, could be considered to lack an essential acidic residue for binding the substrate.

The analytical results on mutations of conserved aspartate and glutamate residues are shown in Table 2. The strictly conserved residues did not include Asp330, which is glutamate in a few species, but the substitution of glutamate for aspartate can be considered conservative. The most striking observation is that all the mutations led to decreased iron and sulfide in the purified variants, and decreased PLP in most of them. Activity could be detected in variants of Glu86, Asp96, Asp165, and Glu236, ruling them out as essential residues required for binding lysine. The acidic residues essential for activity include Asp172, Asp293, and Asp330. In the structure, the side chain-carboxyl groups of Asp293 and Asp330 lie within hydrogen bonding distances to the ϵ -aminium group of the lysyl side chain in the external PLP-aldimine, as illustrated in Fig. 3 (13). Therefore, Asp293 and Asp330 not only interact with the lysyl ϵ -aminium group, but they are essential for activity as well.

Mutations of Asp293 or Asp330, while they inactivate LAM, do not lead to such dramatic decreases in iron and sulfide content as mutation of Asp172 or most of the other conserved residues in Table 2. It seems that the other conserved acidic residues are more important for

the structural integrity of LAM, and for the integrity of the [4Fe–4S] cluster, than either Asp293 or Asp330, which appear to facilitate the proper binding of lysine.

Conserved arginine within and adjacent to the iron-sulfur motif

The aligned amino acid sequences of the known species of LAM show that Arg130 within the motif $_{125}\text{CSMYCRHCTRRR}_{136}$ is conserved in all species, and Arg134 in the adjacent downstream cluster of arginine residues is also conserved in all species of LAM. Arginine135 is present in 37 species, with lysine in its place in 13 other species. Arginine136, present in 26 of 53 species, is least conserved, with glutamate or histidine often appearing in its place.

To examine the significance of the arginine residues we mutated each of them, expressed and purified the variants, analyzed them for activity, iron, sulfide, and PLP content, and obtained the results in Table 3. Mutation of Arg130 or Arg134 to either glutamine or lysine completely abolished enzymatic activity. Mutation of Arg130 also led to dramatically lower iron and sulfide in the purified protein, although PLP binding was little affected. Mutation of Arg134 abolished activity without seriously decreasing the iron and sulfide content.

The importance of Arg130 to the integrity of the [4Fe–4S] cluster suggests a structural role for this residue. The structural graphic in Fig. 4 depicts the likely contribution of Arg130 by showing how the side chain guanidinium moiety is in hydrogen bonded contact with the main chain carbonyl group of Leu55 and the γ -carboxylate of Glu86. The side chain of Arg130 seems, in effect, to cross-link the peptide chain between Arg130 and Leu55, while also constraining the chain at position 86 through a hydrogen bonded electrostatic interaction with Glu86. Further, the main chain carbonyl of Arg130 is hydrogen bonded to the guanidinium group of Arg135, which is also hydrogen bonded to the main chain carbonyl group of Cys132 in the [4Fe–4S] cluster motif. Thus, the arginine claws of Arg130 and Arg135 seem to play important roles in maintaining the structural integrity required for stabilizing the iron-sulfur cluster.

Mutation of Arg134 to glutamine abolishes activity but only slightly decreases the iron, sulfide and PLP content. Mutation to lysine abolishes activity and also substantially decreases the iron, sulfide and PLP content. The structure shows that the carboxylate group of the lysyl-PLP external aldimine is in hydrogen bonded ionic contact with Arg134 (13), and this is apparently essential for activity.

Mutations of Arg135 or Arg136 lead to low but significant activity. Mutation of Arg135 to glutamine significantly decreases the iron, sulfide, and PLP content, but proportionally less than the loss in activity. Mutation of Arg135 to lysine is fairly well tolerated, with modest losses in activity and iron, sulfide, and PLP. Mutation of Arg136 to glutamine leads to dramatically lower iron and sulfide content. Apparently Arg135 is less important than the other arginine residues associated with the iron-sulfur cluster. Arginine136 appears to be important in stabilizing the [4Fe–4S] cluster.

Reconstitution of iron-sulfur clusters

The [4Fe–4S] clusters in LAM are not chemically stable, and chromatography of this protein in the course of purification tends to strip away iron and sulfide. Consequently, the purified enzyme never contains a full complement of iron-sulfur clusters, but they can be reconstituted by incubation of the enzyme with a reducing agent, ferrous sulfate, and sodium sulfide. Reconstitution increases the catalytic activity as well as the iron and sulfide content, as shown in the second line of Table 3. Reconstitution enhances the optical absorption band at 370 nm, as shown in Fig. 5A. Reconstitution of R136Q-LAM also enhances this band and increases the iron and sulfide content, as shown in Fig. 5B and Table 3, but it does not increase the activity.

Reconstitution of R130K-LAM also enhances the iron and sulfide content without increasing activity.

Correlation of structure with functional amino acids

As illustrated in Fig. 6, the conserved arginine and acidic amino acid residues in LAM are located within the β -crescent fold at or peripheral to the active site. They fall into two functional categories, those that bind the ϵ -aminium and carboxylate groups of lysine at the active site, and those that play significant roles in the structural integrity of LAM peripheral to the active site. The former include Arg134, Asp293, and Asp330, which bind the lysyl carboxylate and ϵ -aminium groups. The functional importance of these groups is proven by the present mutagenic results, in which they are shown to be essential for activity, and the points of interaction are proven by the crystal structure (13). These residues do not seem to contribute decisively to the structural integrity of LAM, as indicated by the fact that mutations do not result in major losses of iron, sulfide, or PLP in the purified enzyme.

In contrast, mutations of Arg130 and Asp172 completely abolish activity and destabilize the iron-sulfur centers. These residues are not in direct contact with the substrate or coenzymes and so must play a structural role, as further implied by the very low iron and sulfide contents of the purified variants. Mutations of Glu86, Asp96, Asp165, Glu236 and Arg136 lead to low but readily detectable activities in the variants, so these residues should not be regarded as essential; however, mutations lead to moderate to severe destabilization of the iron-sulfur centers, indicating structural roles. An interaction network among Arg130 and Cys132 (main chain C=O), in the iron-sulfur motif, and Glu86, Arg135, and Leu55 (main chain C=O) shown in Fig. 4 supports the assignment of structural roles to these residues.

The observation of instability in the iron-sulfur centers induced by a mutation is compatible with a structural role for that residue, but it does not prove that the loss in [4Fe-4S] clusters accounts for decreased activity. Thus, reconstitutions of the iron-sulfur centers in R130K- and R136K-LAM do not restore activity (Table 3). Structural perturbations can lead to a myriad of consequences to activity and the binding of coenzymes and prosthetic groups.

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Fig. 1. The mechanism of hydrogen transfer and isomerization in the action of LAM

The internal aldimine of PLP with Lys337 undergoes transaldimination with (S)-lysine to form the external aldimine between PLP and (S)-lysine. Reversible reductive cleavage of SAM generates the 5-deoxyadenosyl radical, which abstracts C3(H_R) from the lysyl side chain to form 5-deoxyadenosine and radical intermediate **1**, which then undergoes isomerization through the azacyclopropylcarbinyl radical **2** to form the product-related radical **3**. The product-related external aldimine of β -(S)-lysine with PLP arises through hydrogen transfer from the methyl group of 5'-deoxyadenosine to radical **3** into the C2(H_R) position, and transaldimination with Lys337 releases β -(S)-lysine.

Fig. 2. Key amino acid residues in highly conserved segments of LAM

The full lengths of 55 LAM sequences were aligned using progressive alignment with the Gonnet PAM 250 protein matrix weights (22) as implemented in the program Clustal_X and Clustal_W (23). The amino acids are depicted in standard letter codes, and the numbering corresponds to the sequence of LAM from *C. subterminale* SB4. The figure emphasizes the residues under consideration in highly conserved segments of the LAM sequence, as illustrated in logo format (24,25). Letters for fully conserved residues span the entire vertical spaces: examples include the cysteine residues 125, 129, and 132 in the iron-sulfur motif characteristic of radical SAM enzymes. Letters for partially conserved residues share vertical space with other residues in proportion to the frequencies with which each appears in the 55 sequences. Thus, arginine appears most frequently at position 135, with lysine appearing less frequently.

Fig. 3. Hydrogen bonded contacts of the lysyl-PLP external aldimine in the structure of LAM
The contacts shown are derived from the x-ray crystal structure of the complex of LAM with (S)-lysine, PLP, and SeSAM (13).

Fig. 4. Roles of conserved arginine residues in stabilizing the fold of LAM

The hydrogen bonded contacts of Arg130 and Arg135 are illustrated structurally in this model. Note how the guanidine group of Arg130 serves to cross-link three chain segments: the hydrogen bond to the main chain carbonyl group of Leu55 links the polypeptide chain between positions 55 and 130, and the hydrogen bonded ionic interaction with Glu86 links the peptide chain between positions 86 and 130. The main chain carbonyl of Arg130 is in turn hydrogen bonded to the guanidinium group of Arg135. This figure was created in SETOR (27) by Cheryl Kreinbring from PDB 2A5H (13).

Fig. 5. Spectral enhancement upon reconstitution of the iron-sulfur center in LAM
A. Spectra of purified LAM before reconstitution (---) and after reconstitution (—). B. Spectra of purified R136Q-LAM before reconstitution (---) and after reconstitution (—).

Fig. 6. Locations of functionally important and conserved acidic and arginine residues in LAM
The structure of a LAM-subunit is illustrated in a ribbon diagram showing the β -crescent (or open barrel) fold. The side chains of amino acid residues mutated in this work are shown as stick models, blue for arginine, red for aspartate, and green for glutamate. These residues appear either in the active site binding the substrate near the cofactors or peripheral to the active site but within the β -crescent. This figure was created in SETOR (27) by Cheryl Kreinbring from PDB 2A5H (13).

Table 1

Plus strand primers used in site-directed mutagenesis.

Mutation	Plus strand primer
R130Q	5'-TGTGCTCAATGTACTGCCAAGACTGTACAAGAAGAAGATTTC
R134Q	5'-GTACTGCAGACACTGTACACAAAGAAGATTTCAGGACAAAGCG
R135Q	5'-GTACTGCAGACACTGTACAAGACAAAGATTTCAGGACAAAGCG
R136Q	5'-GTACTGCAGACACTGTACAAGAAGACAATTTCAGGACAAAGCG
R130K	5'-TGTGCTCAATGTACTGCAAACTGTACAAGAAGAAGATTTC
R134K	5'-GTACTGCAGACACTGTACAAAAAGAAGATTTCAGGACAAAGCG
R135K	5'-GTACTGCAGACACTGTACAAGAAAAAGATTTCAGGACAAAGCG
R136K	5'-GTACTGCAGACACTGTACAAGAAGAAAAATTTCAGGACAAAGCG
E86Q	5'-CAAGCTATTCCAACAGCATTACAGCTTAACAAAGCTGCTGC-3'
D96N	5'-GCTGCTGCAGATCTTGAAAACCCATTACATGAAGATACAG-3'
D165N	5'-GAAATACTCCTCAAGTTAGAAACGTATTATTATCAGGTGGAG-3'
D172N	5'-CGTATTATTATCAGGTGGAACGCTCTTTTAGTATCTG-3'
E236Q	5'-CTCACTTTAACCATCCAAATCAAATAACAGAAGAATCAACTAG-3'
D293N	5'-CCTTACTACATCTATCAATGTAAGTTATCATTAGGACTTGAGC-3'
D330A	5'-GCGTACCAACATTTCGTTGTTGCCGCTCCAGGTGGTGGTGG-3'
D330N	5'-GCGTACCAACATTTCGTTGTTAACGCTCCAGGTGGTGGTGG-3'

Table 2

Mutation of conserved glutamate and aspartate residues in His-tagged LAM.

Mutation	Activity (U mg ⁻¹) ^a	Fe/subunit ^b	S ²⁻ /subunit ^b	PLP/subunit ^b
Wild-type	47 ± 6	2.7 ± 0.3	1.8 ± 0.2	0.70 ± 0.05
E86Q	8.5 ± 0.9	0.50 ± 0.02	0.40 ± 0.05	0.43 ± 0.03
D96N	0.08 ± 0.01	1.2 ± 0.1	0.95 ± 0.10	0.70 ± 0.05
D165N	0.04 ± 0.01	0.23 ± 0.01	0.13 ± 0.02	0.13 ± 0.02
E236Q	0.42 ± 0.03	0.70 ± 0.03	0.52 ± 0.03	0.17 ± 0.01
D172N	ND ^c	0.43 ± 0.02	0.13 ± 0.02	0.11 ± 0.02
D293N	ND ^c	0.97 ± 0.05	0.58 ± 0.03	0.23 ± 0.01
D330A	ND ^c	1.9 ± 0.1	1.3 ± 0.1	0.42 ± 0.07
D330N	ND ^c	1.0 ± 0.1	0.63 ± 0.05	0.60 ± 0.04

^a Assayed by the radiochemical method as described (6).^b Expressed as the number of ions or molecules per subunit as the mean value ± SD.^c No detectable activity.

Table 3

Effects of mutations of arginine residues on the activity and cofactor content of LAM

Mutation	Specific Activity ^a	Fe/Subunit	S ²⁺ /Subunit	PLP/Subunit
WT	64 ± 5.2	3.2 ± 0.07	2.7 ± 0.06	0.97 ± 0.07
WT (FeS) ^b	88 ± 7.9	5.5 ± 0.19	4.9 ± 0.40	0.89 ± 0.04
WT (His) ^c	62 ± 1.7	2.9 ± 0.11	3.4 ± 0.08	0.66 ± 0.03
R130Q	< 0.01	0.42 ± 0.01	0.20 ± 0.02	0.43 ± 0.03
R130K ^c	< 0.01	0.20 ± 0.01	0.30 ± 0.02	0.84 ± 0.05
R130K (FeS), ^{bc}	< 0.01	3.0 ± 0.01	2.9 ± 0.10	0.82 ± 0.02
R134Q	< 0.01	2.8 ± 0.05	2.7 ± 0.10	0.84 ± 0.03
R134K ^c	< 0.01	1.5 ± 0.09	1.5 ± 0.05	0.59 ± 0.03
R135Q	0.98 ± 0.13	1.8 ± 0.02	1.5 ± 0.04	0.58 ± 0.02
R135K ^c	9.9 ± 0.68	2.4 ± 0.07	1.6 ± 0.01	0.77 ± 0.01
R136Q ^d	0.80 ± 0.01	0.48 ± 0.01	0.35 ± 0.02	0.98 ± 0.01
R136Q (FeS) ^b	0.85 ± 0.20	1.9 ± 0.02	1.6 ± 0.17	0.75 ± 0.02

^a Specific Activity (Units/mg protein): Assayed by derivatization of (S)-lysine and (S)-β-lysine the PITC-derivatives followed by HPLC-separation, as described (20,26).

^b Iron-sulfur centers reconstituted.

^c His-tagged

^d R136K - Not expressed