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Radical Stabilization Is Crucial in the Mechanism of Action of Lysine 5,6-Aminomutase: Role of Tyrosine-263α As Revealed by Electron Paramagnetic Resonance Spectroscopy

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Supporting Information

ABSTRACT: Adenosylcobalamin- and pyridoxal-5′-phosphate-dependent lysine 5,6-aminomutase utilizes free radical intermediates to mediate 1,2-amino group rearrangement, during which an elusive high-energy aziridincarbinyl radical is proposed to be central in the mechanism of action. Understanding how the enzyme participates in stabilizing any of the radical intermediates is fundamentally significant. Y263F mutation abolished the enzymatic activity. With isotope-edited EPR methods, the roles of the Tyr263α residue in the putative active site are revealed. The Tyr263α residue stabilizes a radical intermediate, which most likely is the aziridincarbinyl radical, either by acting as a spin-relay device or serving as an anchor for the pyridine ring of pyridoxal-5′-phosphate through aromatic π-stacking interactions during spin transfer. The Tyr263α residue also protects the radical intermediate from interception by molecular oxygen. This study supports the proposed reaction mechanism, including the aziridincarbinyl radical, which has eluded detection for more than two decades.

Adenosylcobalamin (AdoCbl)- and pyridoxal-5′-phosphate (PLP)-dependent lysine 5,6-aminomutase (5,6-LAM) is essential for the metabolism of L-lysine and D-lysine in anaerobic bacteria.[1] It catalyzes a reversible 1,2-shift of the ε-amino group of D-lysine or L-β-lysine into 2,5- or 3,5-diaminohexanoic acid. 5,6-LAM is an $\alpha_2\beta_2$ tetramer. The substrate-free crystal structure of 5,6-LAM shows that AdoCbl is positioned in the β subunit and PLP is anchored in the α subunit and that the distance between them is ~24 Å.[2] Figure 1 shows the PLP binding site. The phenolic oxygen and pyridine nitrogen of PLP interact with Asn299α and Ser238α, respectively, through H-bonding. The 5′-phosphate of PLP is stabilized by interactions with either the side or main chains of a number of neighboring residues. While the network of binding contacts to PLP originates from the α subunit, PLP also binds to the β subunit through an internal aldimine linkage with Lys144β.[2,3] This linkage is important in tethering the two subunits and locking AdoCbl away from the active site in the precatalytic state. Catalysis begins with transaldimination upon lysine binding. Release of the internal aldimine linkage allows the β subunit to rotate, bringing AdoCbl into proximity of the substrate−PLP complex in preparation for the succeeding radical-mediated 1,2-amino group migration.

A plausible radical-based reaction mechanism was proposed by Frey and co-workers (Scheme 1).[3,4] Homolytic cleavage of the

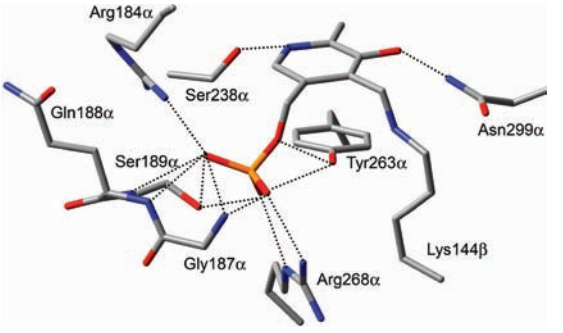


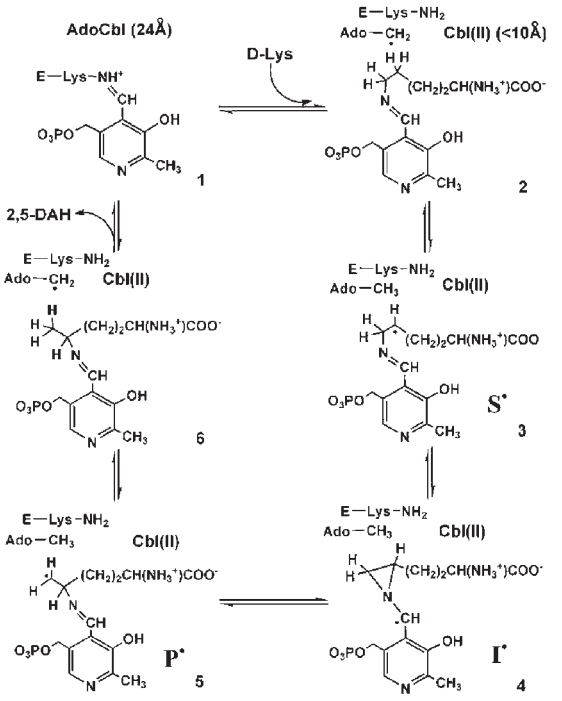
Figure 1. Crystal structure[2] of 5,6-LAM with PLP and AdoCbl, revealing the bonding between PLP and side- and main-chain atoms.

AdoCbl Co−C bond generates 5′-deoxyadenosyl radical (5′-Ado$^\bullet$) and cob(II)alamin. Ado$^\bullet$ abstracts a hydrogen atom from the PLP-bound substrate, forming a PLP−substrate radical (S$^\bullet$). Intramolecular rearrangement proceeds, presumably via a hitherto elusive cyclic aziridincarbinyl−PLP radical (I$^\bullet$), to form a PLP−product radical (P$^\bullet$). P$^\bullet$ reabstracts a hydrogen atom from 5′-deoxyadenosine to form the product, and the 5′-Ado$^\bullet$ recombines with cob(II)alamin to form AdoCbl. Product release, reformation of the PLP−Lys144β internal aldimine, and disengagement of AdoCbl from the active site complete the catalytic cycle. Theoretical energy profiles[5a] for the gas-phase reaction suggested that ring closure of S$^\bullet$ to form cyclic I$^\bullet$ is endothermic by 25 kJ mol^{-1} with a transition-state barrier of 40 kJ mol^{-1}, although PLP might stabilize this radical species by spin delocalization and captodative effects.[5b] An understanding of how the enzyme participates in this chemically unfavorable event is of fundamental importance and great interest.

Figure 1 shows the juxtaposition of the phenol ring of Tyr263α to the pyridine ring of PLP. The two rings, which are oriented in a slipped geometry favoring $\pi-\sigma$ attraction, are tilted by ~15° and separated by an interplanar distance of ~3.8 Å. In addition, the phenolic group of Tyr263α has H-bonding interactions with the PLP phosphate. The fact that Tyr263α is engaged in a stacking interaction with the pyridine ring of PLP, which is proposed to modulate the electron distribution in the highly energetic intermediate I$^\bullet$,[5b] suggests possible roles for this residue in radical stabilization or correctly orienting the PLP at the active

Received: August 17, 2011
Published: September 22, 2011

Scheme 1. Hypothetical Mechanism of the Reaction of 5,6-LAM with D-Lysine



site. In this paper, the role of Tyr263α is explored by site-directed mutagenesis and isotope-edited EPR spectroscopy. Mutation of Tyr263α to Phe263α (Y263F), removing the phenolic bonding contacts with the PLP phosphate, offers flexibility to the benzene ring of Phe263α either to rotate or to swing away from the appropriate geometry for interaction with PLP.

Y263F mutation abolished the enzymatic activity (Figure S1 in the Supporting Information), identifying Tyr263α as a functionally important residue in wild-type (WT) 5,6-LAM. EPR experiments provided insight into how the Y263F mutation disrupts the catalytic cycle of the enzyme. No free radicals could be detected in the reaction of the Y263F mutant with D- or L-lysine, presumably because the radicals are too short-lived to be observed by EPR spectroscopy, as in the case of WT 5,6-LAM. However, the 4-thia analogue of lysine (with sulfur in place of C4) offers the potential to stabilize radical intermediates for EPR detection in WT 5,6-LAM and imitates the early reaction steps in the proposed reaction mechanism.[6]

The X-band EPR spectrum generated from early (<10 s) reaction of the Y263F mutant with 4-thia-L-lysine (Figure 2a) is identical to that generated from WT 5,6-LAM (Figure 2a′). The EPR signal in WT 5,6-LAM was attributed to the presence of a substrate-based radical (termed the transient radical) that is strongly coupled with the low-spin Co^{II} in cob(II)alamin in the active site of the enzyme.[6a] The identical spectral interpretation can be deduced for the Y263F mutant. In AdoCbl- or adenosylmethionine-dependent enzymes,[7] EPR spectroscopy[8] reflects the relative distance and orientations of the two interacting spins and the identity and spin distribution in the component radicals.[9] In the present case, the EPR spectra are identical in line position, line shape, and spin counting as well, suggesting that the PLP binding, the rate of the transimination, the efficiency of protein motion, the propensity for Co—C bond cleavage, the favorable binding geometry of 4-thia-L-lysine and its

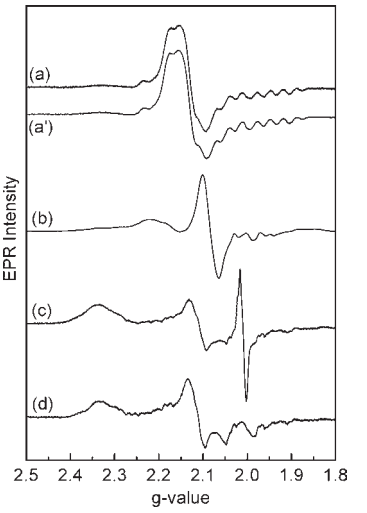


Figure 2. EPR spectra measured at 77 K for radicals generated in (a, c, d) Y263F and (a′, b) WT 5,6-LAM: holoenzyme mixing with 4-thia-L-lysine anaerobically for 8 s (a, a′) and 3 min (b, c) and aerobically for 3 min (d). Instrument settings: microwave frequency, 9.54 GHz; microwave power, 2 mW; modulation amplitude, 4 G at 100 kHz.

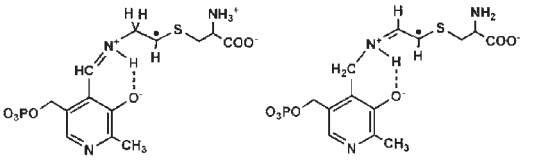


Figure 3. (left) Transient and (right) persistent radical structures.[6a]

distance from AdoCbl, and the efficiency of H-atom abstraction by Ado$^{\bullet}$ are not altered by Y263F mutation. These are essential steps. The integrity of the active site is thus retained in the Y263F mutant at this stage of the reaction.

In WT 5,6-LAM, the transient radical evolves into a more stable radical (termed the persistent radical[6a]) within 3 min. The persistent radical was assigned as a tautomerization product of the transient radical obtained by transfer of a proton between C6 of the 4-thialysyl side chain and the carboxaldehyde carbon (C4′) of PLP (Figure 3).[6a] The persistent radical remains spin-coupled with Co^{II}, giving rise to the EPR spectrum shown in Figure 2b. Amazingly, under exactly the same experimental conditions, the EPR spectrum of the Y263F mutant (Figure 2c) differs considerably from that of WT 5,6-LAM. This immediately suggests that the phenolic group of Tyr263α comes into play during the transformation of the transient radical to the persistent radical in WT 5,6-LAM and also shows that there is a significant interaction between the phenol ring of Tyr263α and the pyridine ring of PLP at the persistent radical stage in WT 5,6-LAM.

The two EPR spectra shown in Figure 2b,c differ in three aspects. First, the major EPR peak of the spin-coupled ensemble in the Y263F mutant is centered at $g = 2.112$ (Figure 2c), whereas in WT 5,6-LAM, the corresponding peak is centered at $g = 2.084$ (Figure 2b). This means that the isotropic exchange coupling constant J and dipole—dipole interaction parameter D of the spin-coupled ensemble in Y263F mutant are larger than those in WT 5,6-LAM (Figure S2 and Table S1). Changes in the microenvironment of the active-site structure could alter the above two parameters. Second, while the persistent radical in WT

dx.doi.org/10.1021/ja207766c |J. Am. Chem. Soc. 2011, 133, 17152–17155

5,6-LAM remains spin-coupled with Co^{II} and is stable for at least 10 min, the spin-coupled ensemble in the Y263F mutant is unstable and decomposes into two separate EPR identities, namely, cob(II)alamin with $g_\perp$ = 2.34 and a radical intermediate at g = 2.006, hereafter termed the second radical intermediate in Y263F. This observation is the first example of spin−spin decoupling in AdoCbl-dependent enzymes. The usual ^{59}Co octet pattern of isolated cob(II)alamin in the $g_\parallel$ region is not resolved because it overlaps harshly with the ^{59}Co induced satellite pattern originating from the spin-coupled ensemble at g = 2.112, a pattern similar to those displayed in Figure 2a,b,d. Third, the sensitivity of the second radical intermediate to air in the Y263F mutant is reflected by the disappearance of the g = 2.006 EPR signal under aerobic conditions (Figure 2d), whereas the EPR signal of the persistent radical in WT 5,6-LAM is not acutely sensitive to air (data not shown). Further analyses of the EPR spectra are given in Figures S3 and S4.

Taken together, the initial changes in the spin−spin magnetic interaction parameters, the spur of the Co^{II}−substrate radical pair spin decoupling, and the eventual oxidative damage to the radical intermediate signify that an improper active-site motion occurs during the transformation of the transient radical to the second radical in the Y263F mutant. Thus, one obvious role of Tyr263α in WT 5,6-LAM is to stabilize the persistent radical, in addition to shielding the radical from interception by O_2.

Previous EPR experiments with ^2H- and 5,6-$^{13}C_2$-labeled 4-thia analogues of lysine proved that the transient and persistent radicals in WT 5,6-LAM are both derived from the substrate and that the spin resides on the C5−C6 locus of the 4-thialysyl side chain.[6a] Since we have shown that Tyr263α comes into play only in the persistent radical stage, we hoped to distinguish further the differences in spin distribution in these radicals generated in WT 5,6-LAM and the Y263F mutant by reacting them with 4-thia-L-[5-^{13}C]lysine and 4-thia-L-[6-^{13}C]lysine.

Figure 4A shows that the line shape and width in the spectrum of the transient radical incorporating 6-^{13}C substitution are the same as those of the natural-abundance 4-thia-L-lysine within the experimental signal-to-noise ratio. Therefore, no significant spin resides on C6. On the other hand, for the radical incorporating 5-^{13}C substitution, although the usual line width broadening from the $I = {}^1/_2$ ^{13}C hyperfine splitting is obscured by the large spin−spin exchange interaction and dipolar broadening at the central peak, obvious line shape changes and line width broadening can be seen in the satellite region. This demonstrates that the spin is predominantly localized at the C5 position of the transient radical and thus that its structure is analogous to that of S$^\bullet$ in Scheme 1.

Figure 4B shows the spectra of the persistent radical in WT 5,6-LAM incorporating either 6-^{13}C or 5-^{13}C substitutions; both exhibit a modest line broadening of ∼5 G on top of the spin−spin dipolar-broadened central peak, relative to the diamagnetic natural-abundance-^{12}C substrate. In addition, the 5-^{13}C substitution introduces further broadening in the ^{59}Co satellite pattern (for example, in the 2.02 > g > 1.97 region). These results demonstrate the presence of spin densities at both the C5 and C6 positions, with C5 as the primary radical center. This observation fully supports the previous assignment[6a] of the tautomerization radical as the persistent radical in WT 5,6-LAM and is also consistent with a previous ^2H and ^{31}P electron−nuclear double resonance (ENDOR) study[6b] of the same radical using isotopically labeled coenzyme [4′-^{2}H]PLP, which showed that spin densities from the C5 radical center are transferred to ^2H and ^{31}P of [4′-^{2}H]PLP. We note that neither an ^2H nor

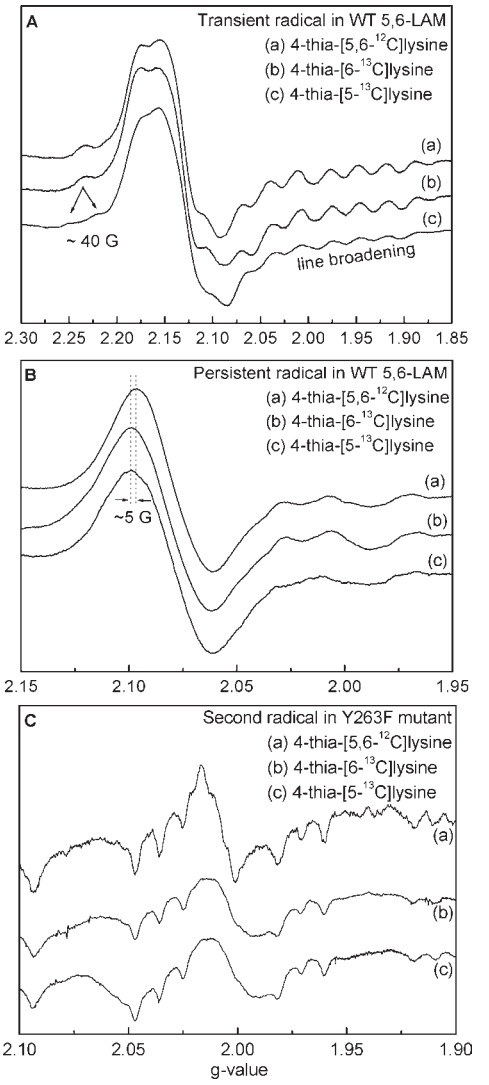


Figure 4. EPR spectra of the radical region of samples of (A, B) holo-WT 5,6-LAM and (C) Y263F mutant at 77 K in reaction with natural-abundance and isotopically labeled 4-thia-L-lysine, as indicated. Instrument settings: microwave frequency, 9.54 GHz; microwave power, 2 mW; modulation amplitude, 4 G at 100 kHz.

^{31}P ENDOR signal can be detected with the transient radical (data not shown), further confirming that the ^{31}P ENDOR signal originates from PLP and not AdoCbl. Taken together, these results establish that these radical intermediates are derived from the substrate and that the major difference between the transient and the persistent radicals in terms of spin distribution is the spin delocalization phenomena observed in the latter radical.

The second radical intermediate in the Y263F mutant, which gives rise to the relatively narrow signal at $g \approx 2.006$ (Figures 2c and 4C), might be a protein-based radical or a second substrate radical uncoupled from Co^{II}. This issue is clarified in Figure 4C. EPR spectra from samples prepared with either 5-^{13}C or 6-^{13}C substitution exhibit broader line widths and decreases in peak-to-trough height by a factor of ∼2 relative to the unlabeled sample as a result of hyperfine interactions with the $I = {}^1/_2$ ^{13}C nucleus. This demonstrates that the second radical intermediate in the Y263F mutant is substrate-derived. Moreover, the EPR spectra derived from 5-^{13}C and 6-^{13}C substitution are indistinguishable,

suggesting that C5 and C6 possess about equal amounts of spin density, analogous to those isotope-edited EPR spectra observed for the persistent radical (Figure 4B) in WT 5,6-LAM representing a spin delocalized species and readily distinguished from that observed for the transient radical (Figure 4A) having spin predominantly localized at a single carbon atom (C5). Neither an ^{2}H nor ^{31}P ENDOR signal can be resolved at $g = 2.006$ (data not shown), so whether PLP remains intact with the second radical intermediate in Y263F or rebinds the enzyme cannot be assured. Although the exact structure of the second radical intermediate in the Y263F mutant is not resolved by the present data, it seems clear that the radical represents a spin-delocalized species, at least onto both the C5 and C6 positions.

Radical stabilization by Tyr263α would also explain why the Y263F mutant cannot function properly to convert D-lysine into 2,5-DAH. In analogy to the normal formation of the 4-thia-L-lysine-based transient radical in the Y263F mutant, the formation of the D-lysine-based substrate radical ($S^{\bullet}$ in Scheme 1) should not be affected by Y263F mutation either, since the active-site integrity is retained awaiting the state of H-atom abstraction by 5′-Ado$^{\bullet}$. Thus, there must be an intermediate state in the subsequent steps that requires Tyr263α to come into play. Formation of such an intermediate state most likely involves transfer of spin densities from the lysyl side chain to PLP; accordingly, the aromatic stacking interaction between the pyridine ring of PLP and the phenol ring of Tyr263α is altered. The hypothetical cyclic aziridincarbinyl−PLP radical $I^{\bullet}$ in Scheme 1 is a central component enabling turnover to proceed. This radical is also a spin-conjugated species, in which withdrawal of a π electron from the primary radical center at C4′ of PLP into the adjacent pyridine ring is realized.[5b] An illustration of the spin distribution of this species in the gas phase and a comparison with those of $S^{\bullet}$ and $P^{\bullet}$ based on B3LYP/6-31G(d,p) calculations are given in Figure S5. In the Y263F mutant, the absence of the phenolic hydroxyl that tethers Tyr263α to PLP allows the benzene ring of Phe263α to rotate or swing away from the appropriate geometry for interaction with the spin-conjugated PLP−substrate radical framework, which disrupts the catalytic cycle and is responsible for the loss of enzymatic activity. Direct observation of the cyclic $I^{\bullet}$ is not possible because it is too high in energy for detectable amounts to accumulate in the active site. The data and analysis presented here are an alternative approach providing strong support for the idea that the rearrangement process involves an aziridincarbinyl−PLP radical intermediate.

The interaction between the cyclic $I^{\bullet}$ and the Tyr263α residue in WT 5,6-LAM possibly includes the following: (1) the spin distribution in the pyridine ring may be modulated either through a stacking interaction with the phenol ring of Tyr263α or through the phenolic group of Tyr263α (i.e., Tyr263α acts as a molecular spin-relay device); (2) the stacking interaction may also help to lock the cyclic $I^{\bullet}$ intermediate in a conformation for the most efficient operation of the enzyme, for example, regulating the relative orientation of the pyridine ring to the strained aziridine ring of $I^{\bullet}$ during spin transfer from C5 of the lysyl side chain to C4′ of PLP. The relative orientation of the two rings modulates the wave function overlap between the singly occupied orbital at C4′ and the N6 lone pair, which is a factor that contributes to the stabilization of $I^{\bullet}$. Of the above, which contributes more to the stabilization of the cyclic $I^{\bullet}$ intermediate cannot be resolved by the data reported herein and must be the subject of further scientific inquiry.

In summary, isotope-edited EPR studies unambiguously support the previously suggested structures of the two radicals generated in the reaction of WT 5,6-LAM with 4-thia-L-lysine. Y263F mutation does not impinge on those early enzymatic steps, including H-atom abstraction from the substrate by 5′-Ado$^{\bullet}$, but it does alter the exchange coupling of the Co^{II} substrate spin-coupled ensemble and destabilizes it when the spin is delocalized from the 4-thialysyl side chain to PLP. The role of radical stabilization by Tyr263α is established and explains the loss of enzymatic activity in the reaction of WT 5,6-LAM with D-lysine. This study provides strong support for the presence of the hypothetical spin-conjugated cyclic aziridincarbinyl−PLP radical, which has not been observed spectroscopically.

■ ASSOCIATED CONTENT

Supporting Information. Sample preparation and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

The National Science Council of Taiwan (Grant NSC-100-2627-M-259 to S.-C.K.) and the U.S. National Institute of Diabetes and Digestive and Kidney Diseases (Grant DK28607 to P.A.F.) supported this research.

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