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Catalytic Roles of βLys87 in Tryptophan Synthase: ^{15}N Solid State NMR Studies

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Abstract

The proposed mechanism for tryptophan synthase shows βLys87 playing multiple catalytic roles: it bonds to the PLP cofactor, activates C4′ for nucleophilic attack via a protonated Schiff base nitrogen, and abstracts and returns protons to PLP-bound substrates (i.e. acid-base catalysis). ε-^{15}N-lysine TS was prepared to access the protonation state of βLys87 using ^{15}N solid-state nuclear magnetic resonance (SSNMR) spectroscopy for three quasi-stable intermediates along the reaction pathway. These experiments establish that the protonation state of the ε-amino group switches between protonated and neutral states as the β-site undergoes conversion from one intermediate to the next during catalysis, corresponding to mechanistic steps where this lysine residue has been anticipated to play alternating acid and base catalytic roles that help steer reaction specificity in tryptophan synthase catalysis.

Keywords

pyridoxal-5′-phosphate; tryptophan synthase; solid-state nuclear magnetic resonance; acid-base catalysis

1. Introduction

The bioactive form of vitamin B_6, pyridoxal-5′-phosphate (PLP), acts as coenzyme in metabolic transformations of amino acids, including transamination, racemization, and α-, β-, γ-elimination/replacement.[1] Early in the study of PLP enzymes, it was shown that reduction of the holo-enzyme by $NaBH_4$, followed by isolation and identification of modified peptide residues, invariably gave a peptide with PLP covalently linked to the ε-amino group of a lysine side chain, thus establishing that PLP is tethered to the active sites of virtually all PLP-dependent enzymes via a Schiff base linkage to this group.[2-13] Confirmation of the lysine linkage came later as X-ray crystal structures of PLP-dependent enzymes began to emerge. Based on the chemistry of the Schiff base functional group exhibited by small molecules in solution, it was deduced that a protonated Schiff base tautomer likely activates C4′ of PLP for nucleophilic attack by amino acid substrates.[3-6, 14-17] Thus, the lysine side chain not only covalently binds the PLP coenzyme, but is

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directly involved in subsequent stages of catalysis. Given the proximity of this residue to the coenzyme active site, it has long been suspected that it continues to play an acid-base catalytic role after its linkage to the coenzyme has been broken.[14-19] X-ray crystal structures show that it is well-positioned in many cases to supply or remove a proton during the enzymatic transformation.[20-24] Yet experimental evidence to support this continued catalytic role is indirect: the X-ray crystal structures generally do not report the protonation states of active site residues, and optical methods are a far less informative reporter on this group once the Schiff base linkage has been broken. Here, we detail the use of ^{15}N solid-state nuclear magnetic resonance (SSNMR) spectroscopy to directly access the protonation state of the catalytic ε-amino group of the active site lysine that binds PLP in tryptophan synthase (TS). This allows us to map out its protonation state at several points in the catalytic cycle. These studies show that the protonation state of the ε-amino group switches between protonated and neutral states as the PLP-substrate complex undergoes conversion from one intermediate to the next during catalysis, and that these changes correlate to mechanistic steps in which this lysine residue has been anticipated to play alternating acid and base catalytic roles.

Tryptophan synthase presents an important paradigm for understanding enzyme structure-function relationships concerned with PLP-enzyme catalysis, including allosteric regulation of substrate channeling, C-C bond scission/bond synthesis, proton transfers to and from carbon centers, and multiple covalent transformations within a common catalytic site. The bacterial TS $\alpha_2\beta_2$ bienzyme complex catalyzes the final two steps in the biosynthesis of L-tryptophan (Scheme 1), and the β-subunit of TS binds PLP in the holoenzyme (referred to as the internal aldimine form) through a Schiff base linkage to the ε-amino group of βLys87. Catalysis at the α-sites involves C-C bond scission to convert indole-3-glycerol phosphate (IGP) to indole and D-glyceraldehyde-3-phosphate (G3P). Catalysis at the β-sites involves the PLP-dependent replacement of the L-Ser hydroxyl by indole to give L-Trp and a water molecule (Scheme 1).[1, 19, 25-27] In Stage I of the multi-step β-reaction, L-Ser is converted to the quasi-stable α-aminoacrylate intermediate, E(A-A), via gem-diamine, E(GD_1), external aldimine, E(Aex_1), and L-Ser quinonoidal, E(Q_1), intermediates. Stage II of the β-reaction is initiated by substrate channeling of indole from the α-site to the β-site through a 25 Å-long tunnel connecting the α- and β- active sites where it makes a nucleophilic attack on the C^{β} of E(A-A) to form a C-C bond and give the first L-Trp quinonoid species, E(Q_2).[20, 27-29] Formation of E(Q_2) is followed by conversion to the second L-Trp quinonoid species, E(Q_3), then the L-Trp external aldimine, and finally to L-Trp via the L-Trp gem-diamine, E(GD_2).

The proposed mechanism for TS shows βLys87 playing multiple catalytic roles: it bonds to the PLP cofactor, activates C4′ for nucleophilic attack via a protonated Schiff base nitrogen, and abstracts and returns protons to PLP-bound substrates (i.e. acid-base catalysis). Experimental support for these integral contributions comes primarily from X-ray crystallography, which identifies βLys87 as the acid-base residue in closest proximity to the coenzyme C4′ and the substrate C^{α} (see Figure 1), and mutagenesis, which shows that βLys87 is critically important for enzyme activity.[19-23, 26, 30-32] However, the proximity of other amino acid residues with acid-base catalytic potential (e.g., His, Glu, Asp) can make the assignment of the acid-base catalytic role(s) ambiguous. Indeed, it now

seems likely that acid-base catalysis in some PLP enzymes requires both the active site Lys and an additional acid-base group.[22, 33] For example, in TS proton transfer at C^{α} is likely mediated by βLys87, but either βLys87 or βGlu109 could act as an acid catalyst facilitating scission of the hydroxyl C-O bond to give the α-aminoacrylate species. Furthermore, βGlu109 is well-positioned to stabilize the developing positive charge on the indole ring during nucleophilic attack of the indole C3 at C^{β} of the α-aminoacrylate species (Figure 1). [34, 35]

Tryptophan synthase catalysis requires conformational changes that switch the α- and β-subunits from inactive, open conformations to active, closed conformations. These conformational gymnastics shuttle potential acid-base catalytic residues (specifically βGlu109) into close proximity of the substrate during catalysis.[26, 28, 36, 37] The open β-subunit conformation of the L-Ser external aldimine intermediate shows that the side chain of βAsp305 hydrogen bonds to the β-hydroxyl of the L-Ser substrate, stabilizing this species. [21, 22, 33, 38, 39] However, in the closed conformation, βAsp305 shifts far away to form a salt bridge with βArg141 and cannot play an acid-base role in catalysis.[21-23, 26, 32, 33, 39, 40] X-ray structures also confirm that βHis115 remains too far from the substrate-coenzyme complex to be an acid-base catalyst.[21-23, 33] Indeed, the X-ray structures of TS in closed conformations reveal that the ε-amino group of βLys87 is always nearest the substrate C^{α}, while βGlu109 is positioned to facilitate charge stabilization at N1 of the indole ring, indicating these two residues play the primary acid-base and charge stabilization roles in the synthesis of L-Trp.[19-22, 27] Further evidence for an essential role of βLys87 in catalysis comes from mutation of this residue to Thr, which renders the β-site inactive. [31] While this variant is still able to form L-Ser and L-Trp external aldimine species by reaction of these amino acids with apo-enzyme and PLP, no interconversion of these intermediates is detected. These findings provide strong support for the involvement of βLys87 as a key acid-base catalyst. Nevertheless, many single amino acid replacements in the β-subunit render the β-site inactive, and therefore the effects of the βK87T mutation are not conclusive.[41, 42]

Recent X-ray crystal structures solved by our group help to disentangle the acid-base roles played by βLys87.[33, 39] The internal aldimine structure (PDB ID: 4HT3, Figure 1A) shows the β-subunit in the open conformation; the ε-amino N of βLys87 is covalently bonded to PLP and is modeled as the protonated Schiff base form. The locations of the side chain groups of βGlu109, βAsp305 and βArg141 are also shown in relation to the PLP ring. Notice that two conformations of the βAsp305 side chain are observed in this internal aldimine intermediate: one folds in toward the active site, the other folds away from the site toward, but distant from, βArg141. In the open β-subunit conformation, the site is solvent accessible and the opening into the site from solution is sufficiently large to accommodate entry/exit of substrate L-Ser and product L-Trp. The site is filled with six crystallographic waters (not shown). The α-aminoacrylate structure (PDB ID: 4HN4, Figure 1B) shows the β-subunit in the closed conformation with a salt bridge between βAsp305 and βArg141. The ε-amino nitrogen of βLys87 and the carboxylate of βGlu109 are depicted as neutral and ionized, respectively. Three crystallographic waters found in the vicinity of C^{β} are also shown. Notice that both the carboxylate of βGlu109 and the ε-amino of βLys87 are sufficiently close to one or another of these waters to qualify as a candidate for catalysis of

water attack on C^{β} of the α-aminoacrylate moiety to give the L-Ser quinonoid species (the reverse of the water elimination step). The 2-aminophenol quinonoid structure (PDB ID: 4HPJ, Figure 1C) also shows the β-subunit in the closed conformation with the side chains of βAsp305 and βArg141 hydrogen-bonded in a salt bridge that stabilizes the closed subunit conformation. 2-aminophenol (2AP) acts as an analogue for indole; reaction of 2AP with the α-aminoacrylate intermediate gives a long-lasting quinonoid intermediate that does not appear to turn over to product (Scheme 2, showing progression from the α-aminoacrylate through a putative $E(Q_2)_{2AP}$ intermediate to the stable $E(Q_3)_{2AP}$ form).[41] The ε-N of βLys87 is shown protonated and located in close proximity (3.9 Å) to C^{α} of the quinonoid intermediate and only 3.0 Å from the 2AP amino group. The waters seen in (B) are displaced by the 2AP group. The amino group of 2AP is bonded to the Ser substrate through an N-C bond formed by nucleophilic attack of the 2AP amino group on C^{β}. The carboxylate of βGlu109 is hydrogen bonded to the phenolic oxygen of 2AP.

While X-ray and mutagenesis studies suggest the acid-base roles of βLys87, atomic-resolution probes such as NMR spectroscopy have not yet been applied to follow the protonation/charge state of the ε-amino nitrogen at multiple points along the catalytic path. The chemical shift in NMR is an extremely sensitive reporter of chemical and charge state, and the ε-Lys-N in the Schiff base is a particularly attractive target, as changes in chemical shift of up to 100 ppm are observed upon protonation or deprotonation.[43] This marker has been used to determine the protonation state of the Lys Schiff base linkage in model compounds,[43-46] the retinal Schiff base in bacteriorhodopsin,[47] and for Schiff base linkages from substrates to PLP in tryptophan synthase[23, 39] and alanine racemase.[48] Upon liberation of the lysine residue, the chemical shift of the ε-amino nitrogen shows a much less dramatic, but nonetheless informative, dependence on protonation state, varying from 33 to 24 ppm when switched from the protonated to neutral forms, respectively.[49, 50] Recently, we reported the ^{15}N chemical shift measurement for the linking imine nitrogen in the internal aldimine state of tryptophan synthase, the first such measurement for the internal aldimine state of a PLP-dependent enzyme.[39] Here we extend this initial work and employ ^{15}N SSNMR combined with enzyme containing ^{15}N enriched ε-amino Lys residues to directly probe the protonation states of βLys87 for two additional stable intermediates in the β-subunit catalytic cycle.

2. Materials and Methods

ε-^{15}N- lysine tryptophan synthase was prepared as previously described through addition of 250 mg ε-^{15}N- Lysine•2HCl to 1 L minimal media during the induction phase of overexpression of *S. typhimurium* TS in *E. coli*, resulting in an enzyme sample in which only the ε-N of each lysine residue contains the NMR-active label.[39] Microcrystals were prepared as described previously[39] and were collected and washed with a 50 mM Cs-bicine solution, pH 7.8, containing 8% PEG-8000 and 1.5 mM spermine, and packed into a Bruker 4 mm magic-angle spinning (MAS) rotor with an approximate volume of 80 μL. When present, serine was introduced by direct addition of 5 μL of 1.2 M L-serine to the packed MAS rotors, while 2AP was introduced by addition of 8 μL of a concentrated stock 2AP in acetonitrile. Solid-state NMR experiments were performed at 9.4 T (400.37 MHz ^{1}H, 40.57 MHz ^{15}N) on a Bruker AVIII spectrometer equipped with an ^{1}H-^{13}C-^{15}N triple

resonance 4 mm MAS probe spinning at a MAS rate of 8 kHz and with the bearing gas cooled to -15 °C, giving an effective sample temperature of -5 °C. Cross-polarization was accomplished at a ^{1}H spin-lock field of 45 kHz, an ^{15}N spin-lock field of 37 kHz (ramped ±2 kHz), and a contact time of 2 ms; 85 kHz Spinal64 ^{1}H decoupling[51] was used throughout. Each spectrum consists of the sum of 81,920 transients acquired with a relaxation delay of 4 s, for a total acquisition time of 3 days 19 h. ^{15}N chemical shifts were referenced indirectly to NH_3(l) via an external solid-state sample of $^{15}NH_4Cl$ (δ[NH3(l)] = 39.3 ppm) calibrated under MAS conditions.

3. Results and Discussion

Observation of the key βLys87 chemical shift in the enzyme active site involves expression of ε-^{15}N- lysine tryptophan synthase. Use of the labeled enzyme in conjunction with strategically labeled ^{13}C,^{15}N- L-serine substrate aids in the determination of the protonation state of βLys87. Furthermore, the protein NMR sample was prepared in the microcrystalline state under catalytically active conditions,[23] making it possible to probe kinetically competent, stable intermediates that lie on or near the reaction coordinate path of the physiological αβ-reaction and their interactions with the βLys87 side chain in three of the intermediates shown in Scheme 1. Stabilization of the active species is made possible through intermediate trapping or by greatly slowing conversion to other species. Trapping arises due to the combined use of low temperature and stabilizing ligands which bind relatively tightly to the α- and β-subunits and stabilize the active, closed αβ-dimeric unit. These ligands consist of N-(4′-trifluoro-methoxybenzenesulfonyl)-2-amino-ethyl phosphate (F9), a tight binding analogue of IGP which binds to the α-site,[21, 22] and Cs^{+}, an analogue of Na^{+}/K,$^{+}$ which binds tightly to the monovalent cation site in the β-subunit.[52-54] The internal aldimine form is the resting state of the enzyme and exhibits an open conformation for the β-subunit. The α-aminoacrylate intermediate is a relatively stable species along the reaction pathway chemically poised to react with indole, but protected from many other nucleophiles, especially water/hydroxide ion, by its sequestration within the confines of the closed αβ-dimeric unit. Even so, its reaction with water to give pyruvate ion and ammonium ion is a significant side reaction.[55, 56] 2AP is an analogue of indole which binds tightly to the β-site and reacts with E(A-A) to form $E(Q)_{2AP}$, the analogue of $E(Q_3)$, which does not turn over to any product.

Figure 2 shows ^{15}N solid-state NMR spectra for TS with F9 bound to the α-site under the following experimental conditions: (A) microcrystals of TS prepared at natural abundance ^{15}N isotopomer concentration; (B) microcrystals of selectively enriched ε-^{15}N-lysine TS; (C) ε-^{15}N-Lys TS microcrystals reacted with 30 mM L-Ser; and (D) ε-^{15}N-Lys TS microcrystals reacted with 30 mM L-[U-^{13}C,^{15}N]Ser and ∼50 mM 2-aminophenol. In Figure 2A, only signals from the large number of protein backbone nitrogens are observed near 120 ppm, while the spectrum of the ε-^{15}N- Lysine TS in Figure 2B reveals a large feature centered at 33 ppm, corresponding to the 26 charged ε-lysine side chains in each αβ subunit. Also evident in Figure 2B is a smaller peak at 202.3 ppm, the expected resonance of a protonated Schiff base. We have previously assigned this resonance to the ε-N of βLys87 using double resonance experiments.[39] ^{15}N SSNMR experiments using ^{15}N-labeled PLP have also established that the pyridine nitrogen is deprotonated in the internal aldimine

form;[39] preliminary data suggest that it remains deprotonated throughout. The observation that βLys87 is indeed protonated in the internal aldimine species confirms a long-standing structural hypothesis for PLP enzymes and is consistent with the mechanistic hypothesis that a protonated imine enhances reactivity toward nucleophiles at C4′, thereby activating catalysis.

Upon addition of L-Ser to the ε-^{15}N-lysine internal aldimine sample, the Schiff base linkage to the βLys87 side chain is broken as stage I of the β-reaction unfolds. Formation of the external aldimine intermediate, conversion to the L-Ser quinonoid species, followed by loss of the β-hydroxyl and a proton from L-Ser produces the next long-lived intermediate in the cycle, the α-aminoacrylate species, E(A-A) (Scheme 1; Figure 1B). The ^{15}N spectrum of E(A-A) shows a significant change: the peak at 202.3 ppm is lost, and a new peak at 24 ppm, indicative of a neutral amine, arises (Figure 2C). These changes indicate that the Schiff base linkage to βLys87 in the enzyme active site has been broken at the same time that a neutral amine has been formed. Based on this correlation, we assign this new resonance to the neutral ε-NH_2 of βLys87. The neutral βLys87 in the α-aminoacrylate intermediate is poised to act as the base in the next stage of catalysis, which consists of proton abstraction from the attacking nucleophile to give the next stable intermediate, the carbanionic quinonoid species.

Reaction of the nucleophile 2AP with the α-aminoacrylate gives the 2AP quinonoid analogue of E(Q_3), (Figure 1C; Scheme 2). The 24 ppm peak of the neutral βLys87 side chain nitrogen now expires, and the only signals detected in the spectrum arise from natural abundance protein backbone amides, centered at 120 ppm, and charged lysine side chains, centered at 33 ppm. To ensure the quinonoid intermediate has indeed formed in situ, the experiment was repeated using ^{15}N-L-Ser. This gave rise to an additional peak at 298.6 ppm (Figure 2D), which is assigned to the Schiff base linkage of the substrate to PLP. The resonance of the Schiff base peak is indicative of a (mostly) deprotonated imine nitrogen and is in good agreement with the previously reported chemical shift of the Schiff base linkage for the indoline quinonoid analogue.[23] The Schiff base peak gives perspective on the expected intensity for the ε-N resonance of βLys87, which appears to have shifted to join the other charged lysine residues centered at 33 ppm. We take these findings as a strong indication that βLys87 is protonated in the quinonoid intermediate state. A protonated ε-amino nitrogen at this stage allows βLys87 to play an effective role as an acid catalyst in the next step of the mechanism, formation of the L-Trp external aldimine (Scheme 1).

These results indicate that βLys87 plays the role of both acid and base during catalysis, activating C4′ and facilitating proton transfers at C^{α} throughout the catalytic cycle. In TS, reaction specificity relies on the ability of the enzyme to fine-tune the microenvironment of the active site to accommodate the charged or neutral βLys87 side chain specifically for each intermediate. It appears that one consequence of these stabilizing interactions is to select the predominant protonation state of the βLys87 ε-amino group for each of the intermediates investigated. Thus the ε-N of βLys87 switches from a protonated Schiff base in the internal aldimine species to a neutral ε-amino group in the α-aminoacrylate intermediate and to a protonated ε-amino group in the 2AP quinonoid species. It is interesting to note that the UV/vis spectrum of the reaction mixture derived from reaction of L-Ser with $\alpha_2\beta_2$ TS shows

a remarkable pH dependence. Yet, the sensitivity to pH arises from a shifting of the β-subunit between open and closed conformations that in turn selectively stabilize the L-Ser external aldimine species (open conformation) and the α-aminoacrylate species (closed conformation). The chemical structures of the active site species do not undergo changes in protonation; the pH dependence arises from deprotonation of protein residues that affect the stability of the open and closed subunit conformations, and, while the distribution of species changes, the protonation states of the intermediates present (internal aldimine, α-aminoacrylate, and quinonoid species) are invariant. This confirms that the active sites are shielded from the effects of bulk solvent over a wide range (pH 6 to 10).[57, 58] The SSNMR results support this finding, as we see a dominant species rather than an equilibrating mixture of protonated and ionized intermediates.

4. Conclusions

The measured ^{15}N chemical shifts of βLys87 in these intermediates provide snapshots of the changing acid-base roles performed by βLys87 during catalysis at the enzyme active site. Initially, the ε-amino group forms a protonated Schiff-base linkage to the PLP cofactor, activating C4′ for nucleophilic addition. Once released, the ε-amino group of the free side chain is found to act as both a base and acid catalyst, with neutral and charged amino groups, respectively. Understanding how the microenvironment of the β-subunit active site fine-tunes the acid-base properties of βLys87, the PLP cofactor, and substrate as the site switches from one intermediate to the next is the subject of our ongoing investigations.

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Abbreviations

PLP	pyridoxal-5′-phosphate
TS	tryptophan synthase
SSNMR	solid-state nuclear magnetic resonance
MAS	magic-angle-spinning
F9	N-(4′-trifluoro-methoxybenzenesulfonyl)-2-amino-ethyl phosphate
2AP	2-aminophenol
IGP	indole-3-glycerol phosphate
G3P	D-glyceraldehyde-3-phosphate
E(A_{in})	enzyme internal aldimine state
E(A-A)	enzyme-substrate α-aminoacrylate intermediate
E(Q)	enzyme-substrate quinonoid intermediate

Highlights

- Protonation state of the active site lysine in tryptophan synthase determined
- ^{15}N solid-state NMR of ε-^{15}N-Lys enzyme
- βLys87 activates C4′ of PLP for substrate nucleophilic attack
- βLys87 abstracts and returns protons to PLP-bound substrates
- The active site lysine ε-amino group plays alternating acid/base catalytic roles

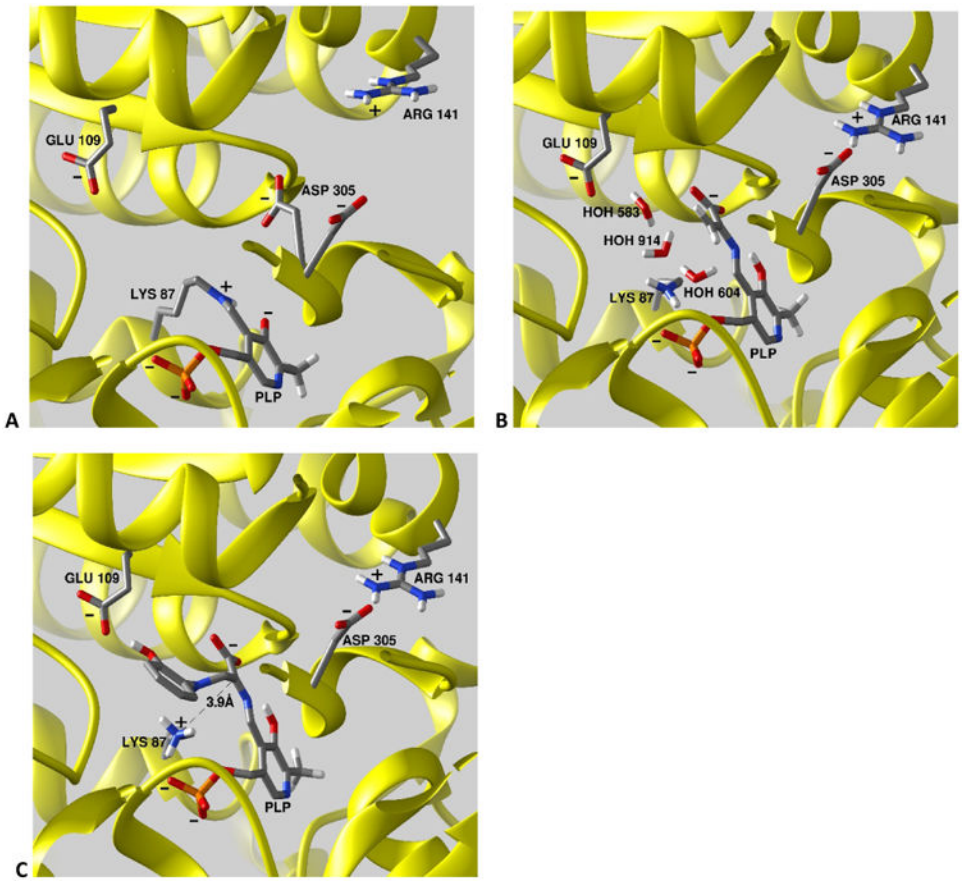


Figure 1.

Comparison of the structures of the β-active site intermediates investigated in this work. (A) the internal aldimine, E(Ain) (PDBID 4HT3); (B) the α-aminoacrylate Schiff base, E(A-A) PDBID 4HN4); and (C) the 2-aminophenol quinonoid, $E(Q)_{2AP}$ (PDBID 4HPJ). Hydrogen atoms have been added to the structure using standard bond lengths and angles, and for βLys87 correspond to the charge states determined in this study. All stick structures are rendered in CPK colors. The β-subunits are depicted as a yellow cartoon ribbon in each panel. For clarity, portions of the β-subunit are cut away to provide views into the β-site. Structures prepared using Chimera.

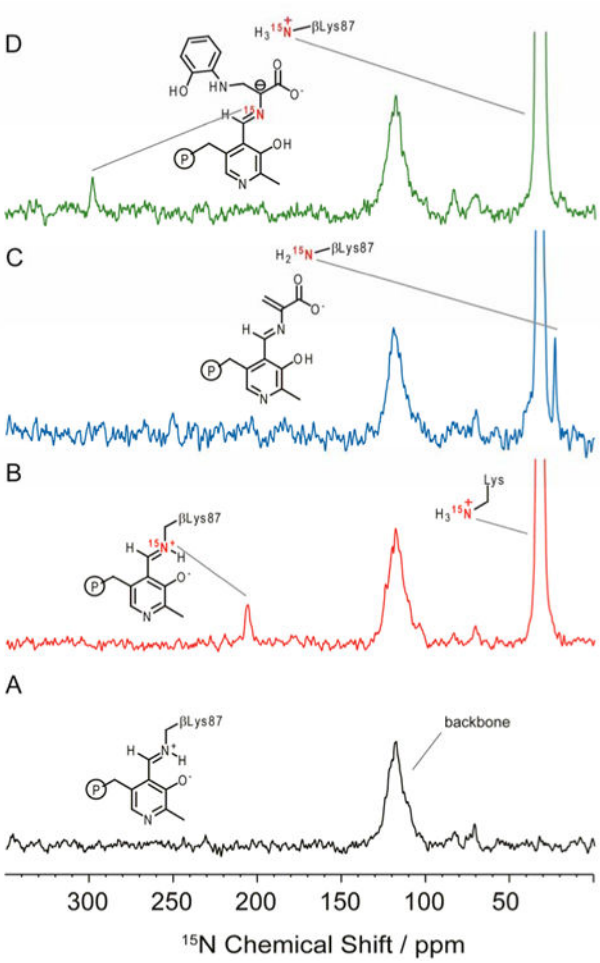
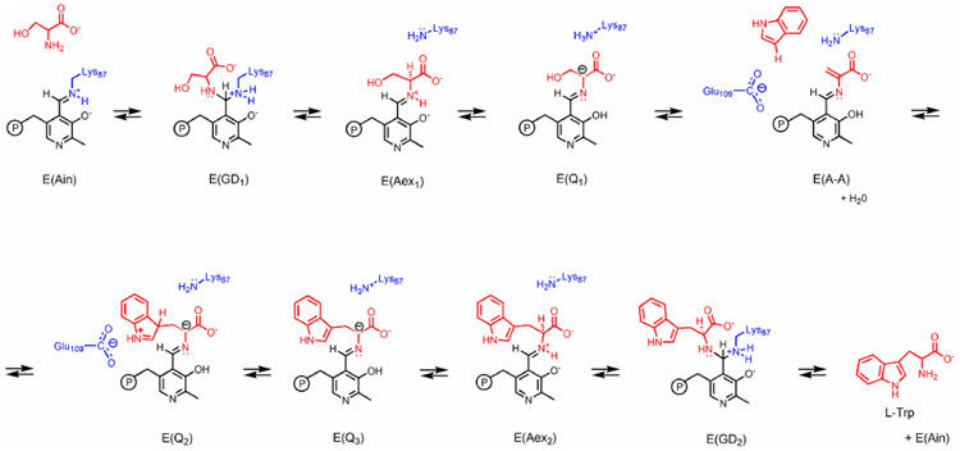
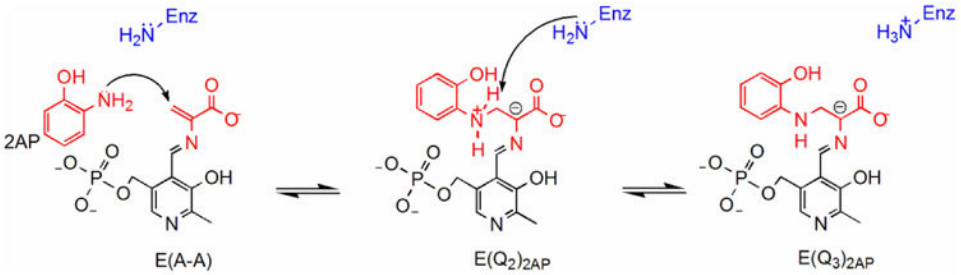


Figure 2.

^{15}N SSNMR cross-polarization magic-angle-spinning (CPMAS) spectra of *S. typhimurium* tryptophan synthase with N-(4′-trifluoro-methoxybenzenesulfonyl)-2-amino-ethyl phosphate (F9) bound to the α-site under the following experimental conditions: (A) Natural abundance ^{15}N isotopomer concentration TS microcrystals, (F9)(Cs^+)E(Ain); (B) ε-^{15}N-Lys TS microcrystals; (C) ε-^{15}N-Lys TS microcrystals reacted with 30 mM L-Ser, giving E(A-A)F9; (D) ε-^{15}N-Lys TS microcrystals reacted with 30 mM L-[U-^{13}C,^{15}N]Ser and ∼50 mM 2-aminophenol, giving (F9)(Cs^+)E(Q_3)$_{2AP}$. Experimental conditions described in Section 2. In (A), only signals from the large number of protein backbone nitrogen atoms are observed, centered at 120 ppm. The spectrum in (B) reveals a large feature at 33 ppm, corresponding to charged lysine side chains throughout the protein, and a resonance at 202.3 ppm that is assigned to the protonated Schiff base linkage from βLys87 to the PLP cofactor. In (C), this linkage has been broken and the ε-amino group of βLys87 is found to be neutral, while in (D), only charged lysine residues are observed.



Scheme 1.
The PLP-dependent β-site reaction of tryptophan synthase.



Scheme 2.
The 2AP reaction.