Direct visualization of critical hydrogen atoms in a pyridoxal 5′-phosphate enzyme

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Enzymes dependent on pyridoxal 5′-phosphate (PLP, the active form of vitamin B6) perform a myriad of diverse chemical transformations. They promote various reactions by modulating the electronic states of PLP through weak interactions in the active site. Neutron crystallography has the unique ability of visualizing the nuclear positions of hydrogen atoms in macromolecules. Here we present a room-temperature neutron structure of a homodimeric PLP-dependent enzyme, aspartate aminotransferase, which was reacted in situ with α-methylaspartate. In one monomer, the PLP remained as an internal aldimine with a deprotonated Schiff base. In the second monomer, the external aldimine formed with the substrate analog. We observe a deuterium equidistant between the Schiff base and the C-terminal carboxylate of the substrate, a position indicative of a low-barrier hydrogen bond. Quantum chemical calculations and a low-pH room-temperature X-ray structure provide insight into the physical phenomena that control the electronic modulation in aspartate aminotransferase.
The vitamin B₆ complex, consisting of pyridoxine, pyridoxal, pyridoxamine, and their phosphorylated derivatives, is involved in neurotransmitter synthesis, amino acid metabolism, glycogen metabolism, and other physiological pathways. Pyridoxal 5'-phosphate (PLP), the biologically active cofactor derived from pyridoxine, is one of the most ubiquitous cofactors found in nature, catalyzing ~140 different types of biochemical transformations. PLP is used in transamination, racemization, phosphorylation, decarboxylation, aldol cleavage, elimination, and replacement reactions. PLP-dependent enzymes are categorized into five recognized fold-types, each performing characteristic chemistry. Such diverse chemical reactions found in PLP-dependent enzymes have intrigued researchers for decades. Fold-type I is the most prevalent, mainly promoting transamination and decarboxylation reactions. Two major hypotheses have been developed to understand the different types of chemistry in PLP-dependent enzymes, namely (1) stereoelectronic control and (2) electronic modulation through selective protonation.

Stereoelectronic control of PLP, also known as the Dunathan hypothesis, involves substrate destabilization by orienting the reactive bond perpendicular to the conjugated π-system in PLP. Alternatively, the hypothesis of electronic modulation through selective protonation asserts that the local active site environment of the enzyme promotes specific protonation states of the cofactor, suggesting that proton positions govern reaction diversity. Thus, the active site protonation states or “protonation profiles” of PLP-dependent enzymes may serve to optimize the desired chemical transformation. The different fold-types of PLP-dependent enzymes induce specific protonation profiles, promoting specific reactions while prohibiting chemically viable side reactions. Verification of the latter hypothesis has been the focus of several NMR studies, but remains elusive. Recent work with NMR crystallography has determined the protonation profiles of PLP in multiple states for tryptophan synthase, a fold-type II PLP-dependent enzyme that promotes β-elimination.

Neutron crystallography is uniquely able to determine all the positions of hydrogen/deuterium (H/D) atoms within a protein. From the coherent nuclear scattering, the nuclear positions of H/D can be directly determined at high to medium resolutions (2.5 Å and higher). Here we report the neutron crystal structure of a PLP-dependent enzyme, aspartate aminotransferase (AAT), a fold-type I PLP-dependent enzyme that reversibly converts l-aspartate and α-ketoglutarate to oxaloacetate and l-glutamate via a ping-pong bi–bi mechanism.

Recombinant porcine cytosolic AAT fortuitously packs as a biological and crystallographic homodimer, with the two active sites having different activities toward the substrate analog α-methylaspartic acid. Thus, in the room-temperature neutron structure of AAT, after the crystal was soaked with α-methylaspartic acid, one site reacts and closes (chain A), whereas the second one remains unreacted and open (chain B). In the open monomer, the Schiff base (SB) is in the internal aldimine form and PLP is covalently linked to a conserved lysine residue (K258). The closed monomer displays the SB in the external aldimine form, with PLP covalently linked to the Cα of α-methylaspartate. Obtaining both states in one crystal enables a direct comparison of the H/D positions in each state (Fig. 1).

To help decipher the impact of protonation, we also determined a low pH, room-temperature X-ray structure of AAT. At pH 4.0, the internal aldimine shows geometric differences near the SB of PLP compared to the neutron structure at pH 7.5. Forced protonation alters the PLP alignment, suggesting that the microenvironment involving Y225 and the phenolic oxygen (O3’) of PLP influence the SB protonation and reactivity. Quantum chemical calculations support the inherent non-coplanarity of the deprotonated SB nitrogen (N₃SB) in the internal aldimine observed in the neutron structure, and suggest that this geometry is stabilized by hyperconjugation, instead of being destabilized by previously proposed strain involving K258. Moreover, our structural analysis provides insight into how PLP enzymes selectively protonate cofactors, substrates, or both during the chemical transformation.
catalytic cycle to promote the desired electronic configurations for specific chemical reactions.

Results

Neutron diffraction. The neutron structure of AAT in complex with α-methylaspartic acid was solved to 2.2 Å resolution (PDB ID 5VJZ) at room temperature (Table 1). Deuterated recombinant AAT was purified and crystallized using hydrogenated reagents. The crystals were exchanged by vapour diffusion with D$_2$O for an extensive period of time (>1 yr), replacing exchangeable hydrons with deuterium. The crystal structure contains both the internal (chain B) and external (chain A) aldimines in the homodimer present in one asymmetric unit. It appears that the two different states were obtained because crystal contacts prohibit one monomer from closing, which is necessary for the enzyme to stabilize a Michaelis complex (Supplementary Fig. 1). The cofactor visualized in the active sites is a mixture of deuterated endogenous PLP and hydrogenated PLP added during crystallization. In both active sites, Y70 side chains act as bridges to the opposing active site, making tight 2.4–2.5 Å hydrogen bonds with the PLP phosphate moieties. R266 (in chains A and B) is cationic, and in a salt bridge with the PLP phosphate. R292(B) and R386(A) are also cationic as they interact with the substrate in the external aldime.

For the internal aldime (chain B), no nuclear density peaks for deuterium were observed near the O$^\prime$ or SB of the PLP cofactor (Fig. 2a). It has been suggested previously that the pK$_a$ of the N$_{SB}$ is ~6.5 for AAT, but estimates vary in the literature (5.4–7.0)\(^{13,15}\). The lack of neutron density for the internal aldime O$^\prime$ deuterium suggests a deprotonated state, which would be stabilized by the PLP resonance and H bonds with O$^\prime$ as well. More importantly, the SB C=N double bond is 46° above the plane of the pyridinium ring, which indicates the lack of a hydrogen bond to O$^\prime$. The out-of-plane geometry for the SB observed in our neutron structure was also identified in other aminotransferase enzymes, with torsion angles ranging from 43 to 96°\(^{13}$. Overall, the experimental evidence demonstrates that O$^\prime$ and the SB are deprotonated and likely carry partial negative charges, stabilized by PLP resonance and H bonds with Y225 and N194.

In the external aldime (chain A), the Y225 hydroxyl adopts the position of a hydrogen bond donor to the PLP O$^\prime$ (2.9 Å) with O$^\prime$ deprotonated similar to the internal aldime (Fig. 3a). Unaltered, N194 remains as a close H bond donor to O$^\prime$. Additionally, K258, which forms the SB in the internal aldime, is neutral in the external aldime active site (Fig. 3c). The newly formed SB in the external aldime adopts a ~28° dihedral angle, positioning the N$_{SB}$ below the PLP pyridinium ring, a 74° difference compared with the internal aldime geometry. A strong nuclear scattering length density peak for a deuterium appears near the N$_{SB}$ in the external aldime (chain A), indicating that the SB is protonated (Fig. 3a). Remarkably, the peak is not between O$^\prime$ and N$_{SB}$, but is instead located between N$_{SB}$ and the C-terminal carboxylate oxygen of α-methylaspartate (Fig. 3a). The N⋯O distance is 2.6 Å and the deuteron is equidistant between the two heteroatoms, with N⋯D and O⋯D distances of 1.5 Å (Fig. 3). The O$^\prime$⋯D distance is 2.5 Å, which is too long to be considered a hydrogen bond, and therefore it cannot contribute to the planarity of the SB and PLP. Thus, the difference in the SB out-of-plane geometries is likely due to changes in the electronics between the internal and external aldime states and not because of hydrogen bond formation between O$^\prime$ and N$_{SB}$.

In AAT, the protonated pyridine nitrogen of the PLP (N1-PLP) enhances charge delocalization through resonance, permitting the subsequent transamination reaction (Fig. 4)\(^{3,16}\). The neutron structure reveals that the N1-PLP is protonated in both the internal and external aldimes (Figs. 2, 3), consistent with previous NMR studies\(^{17}\). In neutron protein crystallography the levels of H/D exchange can be estimated by refining occupancies of the D atoms (see Methods section for details). Interestingly, the D atoms on N1-PLP display different levels of exchange in the two states. In the internal aldime, the N1-PLP hydrogen is essentially fully exchanged with D (D occupancy = 0.91), whereas in the external aldime it is only partially exchanged (D occupancy = 0.45). Thus, the rate of H/D exchange of N1-PLP is reduced in the external aldime state. The observation of the partially exchanged D on N1-PLP in the external aldime may indicate a stronger and less mobile hydrogen bond with D222 than in the internal aldime.

| Table 1 X-ray and neutron crystallographic data collection and refinement |
|-----------------|-----------------|-----------------|-----------------|
|                  | pH 7.5          | pH 4.0          |                  |
|                  | PDB 5VJZ        | PDB 5VZ         |                  |
| **Data collection (neutron)** |                  |                  |                  |
| Beamline/facility | LADI-II/ILL     | LADI-II/ILL     |                  |
| Resolution range (Å) | 55.19–2.20     | 55.19–2.20     |                  |
| Space group       | P2$_1$2$_1$2$_1$| P2$_1$2$_1$2$_1$|                  |
| α, β, γ (°)       | 90, 90, 90      | 90, 90, 90      |                  |
| Completeness (%)  | 72.9 (60.4)     | 72.9 (60.4)     |                  |
| I/ε               | 6.5 (4.9)       | 6.5 (4.9)       |                  |
| R$_{merge}$       | 0.142 (0.186)   | 0.142 (0.186)   |                  |
| Redundancy        | 2.9 (2.4)       | 2.9 (2.4)       |                  |
| **Data collection (X-ray)** |                  |                  |                  |
| Beamline/facility | Rigaku HighFlux | Rigaku HighFlux |                  |
| Resolution range (Å) | 50.00–2.00      | 50.00–2.00      |                  |
| Completeness (%)  | 95.5 (93.9)     | 95.5 (93.9)     |                  |
| I/ε               | 28.3 (5.2)      | 28.3 (5.2)      |                  |
| R$_{merge}$       | 0.031 (0.470)   | 0.031 (0.470)   |                  |
| Redundancy        | 6.3 (6.3)       | 6.3 (6.3)       |                  |
| **Joint X/N refinement** |                  |                  |                  |
| Resolution (neutron, Å) | 20–2.20         | 20–2.20         |                  |
| Resolution (X-ray, Å) | 20–2.20         | 20–2.20         |                  |
| Data rejection criteria |                  |                  |                  |
| No observation and I/ε = 0 |                  |                  |                  |
| Sigma cutoff      | 2.5             | 2.5             |                  |
| No. reflections (neutron) | 32,558          | 32,558          |                  |
| No. reflections (X-ray) | 55,493          | 55,493          |                  |
| R$_{work}$/R$_{free}$ (neutron) | 0.234/0.256     | 0.234/0.256     |                  |
| R$_{work}$/R$_{free}$ (X-ray) | 0.197/0.220     | 0.185/0.225     |                  |
| No. of atoms      | 12,978          | 12,978          | 6429             |
| Protein including H and D | 81              | 81              | 48               |
| Cofactor/ligand   | 735 (245 D$_2$O) | 735 (245 D$_2$O) | 232 (232 O atoms) |
The H bond network consisting of D222, H143, T139, H189, and a cluster of water molecules (Supplementary Fig. 2) is coupled from N1-PLP to the bulk solvent. This H bond network significantly increases the pK<sub>a</sub> of N1-PLP (>7.0) in the active site of AAT compared to 5.8 in solution<sup>19</sup>. Moreover, in the internal aldimine, H143 and H189 are neutral, while in the external aldimine H143 remains neutral but H189 becomes protonated and positively charged (Fig. 5). Our neutron structure agrees with<sup>3</sup>H NMR experiments<sup>20–22</sup> and quantum chemical cluster models of the active site<sup>16</sup>, which showed that H143 and H189 are uncharged (singly protonated) in the internal aldimine state. In the external aldimine, H189 becomes doubly protonated. Furthermore, the electrostatic contribution of H189 was shown to affect the rate of catalysis in AAT, with a 75% decrease in <i>k<sub>cat</sub></i> for the H143L:H189L double mutant compared with a 50% decrease for the H143L single mutant<sup>12</sup>. Thus, as more electron density is added in the external aldimine state, additional positive charge is perhaps necessary to counterbalance the negative charges from the carboxyl groups of the substrate. Due to H189 protonation, the water molecule (D<sub>2</sub>O) that is H bonded to N<sup>81</sup> of H189 changes its relative orientation in the two states (Fig. 5). A cluster of three water molecules (D<sub>2</sub>O) coupled to H189 through H bonds (Supplementary Fig. 2) leads to the bulk solvent through a second cluster of water molecules (D<sub>2</sub>O). The water molecules in the second cluster are not directly connected by H bonds to those in the first cluster, but are more mobile with high B-factors. Thus, they can rearrange to promote a proton transfer by the Grothuss proton hopping mechanism to shuttle protons in and out of the active site (Fig. 5c). This water channel is perhaps responsible for H189 protonation when the external aldimine is formed and may also assist in N1-PLP protonation when the apoenzyme reacts with PLP to generate the internal aldimine.

**Low-pH X-ray structure.** To probe the structural changes that occur upon internal aldimine SB protonation, we obtained a low-pH X-ray structure of AAT in the absence of substrate, in which both chains are in the internal aldimine state. Acidifying AAT crystals with acetic acid vapour, using a procedure described previously<sup>23, 24</sup>, yielded a 1.9 Å room temperature X-ray structure at pH ~4.0 (PDB ID 5VK7; Table 1). When the geometries of the internal aldmines (chain B) from the pH 7.5 and pH 4.0 structures were compared, two major differences were evident. The torsion angle between the SB C = N bond and the pyridinium ring, and the O<sup>″</sup>−O distance between the Y225 hydroxyl and O3′ of PLP are different (Fig. 6). In the pH 4.0 structure, the SB is 22° above the pyridinium ring plane, compared to 46° in the internal aldimine at pH 7.5 (Fig. 6). Additionally, in the pH 4.0 structure the O<sup>″</sup>−O distance between the Y225 hydroxyl and O3′ of PLP is 2.9 Å, compared with 2.6 Å in the internal aldimine at pH 7.5 (Fig. 6). In the other monomer of the pH 4.0 structure (chain A, with restricted motion), the corresponding torsion angle and the (Y225)O<sup>″</sup>−O(O3′) distance are 26° and 3.0 Å, respectively. Protonation of the SB nitrogen of the internal aldimine at low pH appears to introduce an H bond between O3′ and the SB by reducing the torsion angle between SB and the pyridinium ring. However, protonation of O3′ cannot be ruled out as H atoms were not observed in the low pH X-ray structure.

**Quantum chemical calculations.** Previous studies have suggested that the out-of-plane geometry of the internal aldimine SB is
induced by strain caused by the side chain of K258. This strain was proposed to cause ground state destabilization of the cofactor, thus contributing to the “catalytic power” of the enzyme. To probe the nature of this geometric distortion intrinsic to the internal aldimine intramolecular interactions, we performed density functional theory (DFT) calculations using a truncated Lys-PLP model in isolation from the active site (Methods section). Using the protonation states observed in the neutron structure, unconstrained geometry optimization of the internal aldimine model resulted in a C3–C4–C4′–NSB torsion angle of 42°, in excellent agreement with the neutron structure. This finding suggests that the non-coplanarity of the two PLP moieties cannot be explained solely by strain, and that intramolecular electronic forces play an important role. Upon NSB or O3′ protonation, the SB-to-pyridinium torsion angle becomes essentially 0° (Supplementary Fig. 3), resulting from the presence of the incipient NSB...O3′ hydrogen bond. This finding is consistent with the reduced torsion angle of 22° in the pH 4.0 X-ray structure, in which we expect NSB to be protonated. The deviation in the torsion angle between the DFT model and the low-pH structure can be attributed to geometric restraints imposed by the active site that are not present in the DFT models. For example, the π−π stacking interaction between the pyridine ring of PLP and W140 were excluded in the simplified model. Nevertheless, the intramolecular orbital interactions can be considered primary (first-order) effects, whereas noncovalent interactions with nearby residues are second-order. Interestingly, when the N1-PLP and N5′ are both deprotonated in the DFT model, the SB double bond is coplanar (3°) with neutral pyridine (Supplementary Fig. 3), suggesting that the electron-withdrawing capabilities of the pyridine ring influence the SB torsion angle. Using natural bond orbital analysis, we found that the distortion from planarity arises from electronic effects that include electron repulsion between the NSB and O3′ lone pairs, and hyperconjugation (Fig. 7). Hyperconjugation is a stabilizing interaction in which the electrons in either a σ or π-bond are delocalized into an empty or partially filled antibonding σ or π-orbital (σ* or π*). The π → π* interaction energies of the πSB → πC3–C4 and π C3–C4 → π′SB are 5.2 and 12.3 kcal mol−1, respectively (Fig. 7), stabilizing the out-of-plane conformation by 17.5 kcal mol−1. The presence of the hyperconjugative interactions offsets the disruption of conjugation caused by lone-pair repulsion. Similar interactions are present in biphenyl, which has maximal hyperconjugation interactions of ~8.0 kcal mol−1 and a phenyl-to-phenyl torsion angle of 44°. Thus, the interplay of favorable conjugative and hyperconjugative interactions in the internal aldimine contribute to offsetting the lone-pair repulsion (Fig. 7), allowing the SB to adopt the observed non-
cotplanar structure. In the protonated SB model, hyperconjugative interactions between NSB–C4' and C3–C4 significantly decrease, to <0.5 kcal mol\(^{-1}\). A strong hydrogen bond forms between NSB and O3', which allows adoption of a more planar structure. When the N1-PLP is deprotonated the π–π conjugation must increase, stabilizing the SB to be coplanar with the ring despite unfavorable lone-pair repulsion. In both the internal and external aldmines, the SB planarity and reactivity can be tuned by PLP-N1 protonation, a demonstration of electronic modulation by selective protonation. All donor–acceptor orbital interactions were computed with a threshold of 0.5 kcal mol\(^{-1}\) (Supplemental Information).

**Discussion**

The diverse chemistry performed by PLP-dependent enzymes has been poorly understood and was only recently revised through insight from NMR studies\(^6\)–\(^9\). Historically, many of the protonation states of PLP and active site residues were assigned based on spectral analysis or chemical intuition from X-ray structures. The determination of the protonation states by either NMR or neutron crystallography has the power to answer many questions in enzymology and structural biology.

The proposed mechanisms of PLP-catalyzed transamination reactions require a neutral α-amino on the substrate for external aldmine formation. The neutron structure shows that the internal aldmine NSB is deprotonated near-physiological pH (Fig. 2). Therefore, the amino acid substrate may bind initially as a zwitterion in the Michaelis complex and the internal aldmine nitrogen could then accept a proton from the N-terminal NH\(_3^+\) of the substrate, thereby activating the cofactor (Fig. 2b). The bond between PLP and the conserved lysine is cleaved during the formation of the external aldmine (Fig. 4). We found a deuteron positioned equidistant between the NSB nitrogen and oxygen of the substrate C terminus oxygen in the external aldmine, indicative of a low-barrier hydrogen bond (LBHB). In an LBHB, the proton can move back and forth between the two heavy atoms because the zero-point vibrational energy is above the potential energy barrier\(^29\), \(^30\). Here, the formation of the intramolecular LBHB between the NSB and carboxylate oxygen was unexpected. Historically, a double-well hydrogen bond would be expected to have formed between the NSB and O3' of PLP\(^3\).

Previous quantum mechanics/molecular mechanics (QM/MM) calculations suggested that the tautomeric equilibrium between the NSB and O3' in AAT is shifted toward the NSB by >7.0 kcal mol\(^{-1}\). However, to our knowledge, the LBHB (Fig. 3) observed in our neutron structure in the external aldmine has not been detected or proposed for any PLP-dependent enzyme. Interestingly, previous studies have suggested that if the carboxylate oxygen, O3', or both are protonated in PLP-glycine adducts, the pK\(_a\) of the α proton would be significantly decreased, making it more acidic\(^4\), \(^32\). The D atom present in the LBHB in our neutron structure would be highly polarized, containing an increased partial positive charge. Such intramolecular hydrogen bonding may also be present in other PLP-dependent enzymes, in which stabilization of the carbanion (an azomethine ylide) is needed for partial positive charge. Such intramolecular hydrogen bonding may also be present in other PLP-dependent enzymes, in which stabilization of the carbanion (an azomethine ylide) is needed for partial positive charge. Specific chemistry. The presence of the LBHB is further supported by the SB being in the plane of the carboxylate oxygen, making the SB go 28° below the plane of the pyridinium ring rather than being coplanar with O3'.

In the external aldmine the ε-amine of K258 has been proposed to act as a general base that abstracts the α proton from the external aldmine, leading to the formation of a carbanionic intermediate (Fig. 4). The α deprotonation is partially rate-limiting in AAT\(^1\)–\(^3\). In the case of the α-methylaspartate substrate used in this study, the reactive H on α of aspartate has been substituted with a methyl group, precluding the advancement of the AAT-catalyzed reaction beyond the external aldmine state. The neutron structure shows that K258 is neutral (ND\(_2\), Fig. 3c), corroborating its role as the general base catalyst\(^33\). Thus, the proton inventory remains constant between the internal and
external aldimines in our model system: two protons from the protonated α-amino group of the substrate are transferred to K258 and one proton remains on the nascent NSB when the external aldimine is formed.

A recent NMR study has shown that the internal aldimine of tryptophan synthase has a protonated NSB and a deprotonated N1-PLP. In AAT, N1-PLP is protonated in both the internal and external aldimine forms. AAT catalyzes transaminations, whereas tryptophan synthase performs β-eliminations. The difference in the local active site environments of AAT and tryptophan synthase is responsible for the different observed protonation profiles. Specifically, in AAT, an Asp residue forms a salt bridge with N1-PLP, while in tryptophan synthase a Ser residue is hydrogen bonded to N1-PLP. The different protonation states observed in the two enzymes may be responsible for promoting these two specific types of chemistries and preventing side reactions.

We now summarize our observations and calculations as they apply to the transamination reaction as follows:

1. We propose that when the apo-enzyme reacts with PTP to form the internal aldimine, the hydrogen bond network spanning from N1-PLP to the bulk solvent through D222, H143, T139, and H189 is responsible for the N1-PLP protonation via the Grothuss proton hopping mechanism (Fig. 5c)12. N1-PLP protonation influences the coplanarity of the SB relative to the pyridinium ring, with the SB C=N torsion angle tilted by 46°. In addition, N1-PLP protonation is important for the enhanced electron sink effect for stabilization of the carbanion intermediate.

2. O3′ is not protonated in either the internal or external aldimines at near-physiological pH. The negative charge on O3′ is stabilized by resonance, leading to partial negative charges on O3′ and the NSB in the internal aldimine. In both aldimines the negative charge on O3′ is further stabilized by hydrogen bonds with Y225 and N194.

3. NSB is not protonated in the internal aldimine, but is ready to accept a proton from the incoming N-terminal NH₄⁺ group of the substrate aspartate. Deprotonation of the substrate NH₄⁺ activates it to attack the SB carbon and proceed to external aldimine formation. As the protonated NSB switches linkage from K258 to the substrate, the SB C=N bond rotates below (~28°) the pyridinium ring to H bond with the C-terminal carboxylate of the substrate.

4. In the external aldimine, a deuterium is positioned midway between NSB and the C-terminal carboxyl oxygens of α-methylaspartate, participating in an apparent LBHB. This LBHB may stabilize the subsequent formation of the carbanion after Coα is deprotonated by K258 by "shielding" the negative charge of the carboxylate from the Cx carbon.

5. H189 is neutral in the internal aldimine, but becomes protonated, presumably through the water channel, in the external aldimine. The resultant extra positive charge is an additional counterbalance to the negative charge on Coα in the ensuing carbanion.

The direct determination of the hydrogen positions in AAT explains how PLP-dependent enzymes promote specific protonation states and change hydrogen bonding configurations between intermediate steps in the transamination catalytic cycle. As conformational and electrostatic changes are invoked during cofactor and substrate binding, several proton transfers occur to modulate the required electronic configurations. Nature has fine-tuned biochemical reactions by stereoelectronic control and electronic modulation through selective protonation within the active sites of PLP-dependent enzymes.
the geometry optimizations. Tight SCF and optimization convergence criteria were used. Following geometry optimization, vibrational frequencies were computed at the same level of theory to ensure that each geometry corresponds to a local minimum. The internal aldime models were truncated at Cβ of K258, and the phosphate group was removed and replaced by a methyl group (Supplementary Data 1–4). Natural bond orbital (NBO) analysis and second-order perturbative orbital interaction energy analysis were performed with the program NBO 6.0.34. For the NBO analysis only, the C3–C4–C4′−N4β torsion angle was constrained during the optimizations of the models of the internal aldime at pH 7.5 (deprotonated N4β) and pH 4.0 (protonated SP), and the Kohn–Sham density matrix was used for the NBO transformation (Supplementary Data 5, 6). All calculations were performed with Gaussian 09u.

Data availability. Coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 5V1Z and 5VK7. Other data are available from the corresponding authors upon reasonable request.

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S.D., T.C.M., and A.Y.K. conceived and coordinated the study. S.D., T.C.M., and A.Y.K. wrote the paper. S.D., R.C.J., and J.M.P. contributed to the computational analysis. S.D., T.C.M., and A.Y.K. performed the structural analysis. M.P.B. and D.A.K, conducted the neutron data collection. S.D., O.G. and K.L.W, contributed to deuterated protein preparation. The manuscript was written through the contributions of all authors.

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