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Pyridoxal 5'-Phosphate: Electrophilic Catalyst Extraordinaire

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Abstract

Studies of nonenzymatic electrophilic catalysis of carbon deprotonation of glycine show that pyridoxal 5'-phosphate (**PLP**) strongly enhances the carbon acidity of α -amino acids, but that this is not the overriding mechanistic imperative for cofactor catalysis. Although the fully protonated **PLP**-glycine iminium ion adduct exhibits an extraordinary low α -imino carbon acidity ($pK_a = 6$), the more weakly acidic zwitterionic iminium ion adduct ($pK_a = 17$) is selected for use in enzymatic reactions. The similar α -imino carbon acidities of the iminium ion adducts of glycine with 5'-deoxypyridoxal and with phenylglyoxylate shows that the cofactor pyridine nitrogen plays a relatively minor role in carbanion stabilization. The 5'-phosphodianion group of **PLP** likely plays an important role in catalysis by providing up to 12 kcal/mol of binding energy that may be utilized for transition state stabilization.

Introduction

Scientists prize the rush of adrenalin that comes with making an original discovery, or with bringing order to seemingly disconnected experimental observations. Snell and Braunstein must have received tremendous satisfaction from their independent discovery, more than sixty years ago, that heating pyridoxal 5'-phosphate (**PLP**) with an amino acid yields the products of transamination of the amino acid [1]. These results led rapidly to a broad outline of the role of **PLP** in cellular processes. This outline has been expanded and refined in work by a large number of investigators, some of which has been previously reviewed in this journal [2–5].

All biochemists and chemical biologists now have a passing knowledge, or better, of the many enzymatic reactions catalyzed by **PLP**, and an appreciation of the elegant design that enables this small molecule catalyst to labilize several types of bonds at α -amino carbon [6,7]. The wide range of reaction types catalyzed by **PLP** has resulted in its recruitment by an enormous number of enzymes. As of 2004, the enzyme commission has assigned more than 140 EC numbers to **PLP** enzymes, and free living prokaryotes devote ca. 1.5% of their open reading frames to these proteins [8,9]. We have worked in recent years to update and increase our understanding of the chemical mechanism for electrophilic catalysis of carbon deprotonation by **PLP** and by simple ketones.

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Electrophilic Catalysis by Acetone

Our interest in catalysis by **PLP** began when we found that the small tertiary amine base 3-quinuclidinone (**Q**, Figure 1) acts as a bifunctional electrophilic *and* general base catalyst of carbon deprotonation of glycine methyl ester (**GlyOMe**) in aqueous solution [10]. We proposed that this bifunctional catalysis results from formation of an iminium ion adduct between the amino group of **GlyOMe** and the carbonyl group of **Q**, and that the iminium ion then undergoes deprotonation at the α -imino carbon by a second molecule of **Q**. Monofunctional general base and electrophilic catalysis were characterized separately in a study of general-base-catalyzed deprotonation of the α -imino carbon of the iminium ion adduct of **GlyOMe** with the simple ketone acetone in D₂O (Figure 1) [11]. Carbon deprotonation was followed by monitoring the exchange for deuterium of the α -amino protons of glycine using ¹H NMR methods developed in our studies of proton transfer from simple carbon acids in aqueous solution [12,13]. The catalytic power of acetone arises from the large 7-unit decrease in the carbon acid pK_a of 21 for the α -amino carbon of N-protonated **Gly-OMe** [14,15] to a pK_a of 14 for the α -imino carbon of the acetone-**GlyOMe** iminium ion adduct [11]. We were surprised by the large effect of a simple ketone electrophile on carbon acidity, for which there was little or no precedent in the chemical literature.

One defining catalytic property of **PLP** is the large stabilization of the resulting α -imino carbanions (quinonoids), which may be generated by deprotonation [16], decarboxylation [17] or retroaldol cleavage [5] reactions of α -amino acids (Figure 2). It is logical to attribute this large carbanion stabilization to the pyridinium ion electron sink [18]. However, our observation that the simple ketone acetone is also a strong catalyst of deprotonation of α -amino carbon [11] prompted us to examine this assumption by comparing the effect of **PLP** on carbon acidity with the effect of other simple ketones.

An Interesting Diversion

Pyridoxal analogs are effective catalysts of the transamination [19] and racemization [20] reactions of alanine in water at neutral pH. We therefore *expected* that the **PLP** analog 5'-deoxypyridoxal (**DPL**) would be an effective catalyst of proton transfer from the α -amino carbon of glycine, which we planned to detect by monitoring exchange of the α -amino protons of glycine for deuterium from solvent in D₂O [15,21]. We were at first mystified by our failure to detect any deuterium exchange into glycine upon prolonged (several days) incubation of 100 mM glycine with 10 mM **DPL** in D₂O at pD 7.0. Analysis using ¹H NMR spectroscopy revealed the first-order disappearance of **DPL** to give an equilibrium mixture containing 3% **DPL** along with 97% of the diastereomeric products of Claisen-type addition of glycine to **DPL** in a ratio of 2:1, but no detectable (< 1%) incorporation of deuterium from D₂O into glycine or transamination to give 5'-deoxypyridoxamine [22]. The mechanism for formation of the Claisen-type adducts of glycine with **DPL** is shown in Figure 3A. This is not a minor side reaction: there is substantial conversion of **DPL** to the Claisen-type adducts in a reaction that is apparently first-order in **DPL**, even when the initial concentration of **DPL** is as low as ca. 0.1 mM. This reflects the >10,000-fold higher reactivity of the **DPL**-stabilized glycine carbanion (**DPL=Gly⁻**, Figure 3A) towards the carbonyl group of **DPL** (bimolecular reaction) compared with its protonation by solvent water (first-order reaction), so that $k_{\text{add}}/k_{\text{p}} > 10,000 \text{ M}^{-1}$ (Figure 3A). The Claisen-type addition of glycine to pyridoxal was reported more than 50 years ago in a study that focused on the role of metal cations in **PLP**-catalyzed reactions [23]. Claisen-type adducts are also formed from the reaction of aminomalonate with **DPL** where an iminium ion intermediate undergoes decarboxylation to give the **DPL**-stabilized glycine carbanion (**DPL=Gly⁻**, Figure 3A) which then reacts with the carbonyl group of a second molecule of **DPL** [24].

Bimolecular Claisen or aldol condensation reactions are problematic in the acidic protic solvent *water*, in part because Brønsted acids in water are usually more reactive electrophiles than the simple carbonyl group. Even in the case of favorable intramolecular aldol condensation reactions, bimolecular protonation of an acetone-like enolate by buffer acids is significantly faster than intramolecular addition of the enolate to a benzaldehyde-type carbonyl group [25, 26]. The extensive resonance stabilization of **DPL=Gly**[−] apparently favors addition of this *soft* carbon nucleophile to the *soft* carbonyl electrophile **DPL**, rather than its reaction with Brønsted acids which are *hard* electrophiles. In other words, there is a relatively small Marcus intrinsic barrier for nucleophilic addition of **DPL=Gly**[−] to the carbonyl group of **DPL** in water [27].

Enzymes such as serine palmitoyl transferase [28] and 5-aminolevulinate synthase [29] catalyze Claisen-type addition reactions that involve addition of PLP-stabilized α -amino carbanions to thioesters. For example, the key step for the 5-aminolevulinate synthase-catalyzed reaction is addition of the PLP-stabilized glycine carbanion to succinyl-CoA to form a β -keto acid (Figure 3B), which then undergoes decarboxylation to 5-aminolevulinate. Our results show that while the glycine carbanion is strongly stabilized by interactions with the PLP cofactor, it maintains a high kinetic reactivity, so that the key condensation step probably requires no assistance by the enzyme beyond orientation of the carbanion and thioester at the active site.

At high pH, **DPL** is essentially quantitatively converted to its iminium adduct with glycine **DPL=Gly**, and the novel products of addition of **DPL=Gly**[−] to this iminium ion are observed (Figure 4A) [30]. The **DPL**-stabilized alanine carbanion undergoes reaction with **DPL** at its α -pyridyl carbon (Figure 4B), presumably because the methyl group at the α -imino carbon creates steric hindrance to the reaction at this position [31]. These unexpected reactions of **DPL**-stabilized amino acid carbanions with **DPL** reveal an intriguingly high chemical affinity of these carbanions for the carbonyl group. To the best of our knowledge these condensation reactions are scrupulously avoided in the cell, in order to preserve the precious **PLP** cofactor.

Substituent Effects on Carbon Acidity

Figure 5 reports the carbon acid pK_a for glycine zwitterion ($pK_a = 29$) [15] and for several iminium ion adducts of glycine [11,32,33]. This Figure shows the following:

- (1) The dominant form of the **DPL**-glycine iminium ion adduct at pH 7 is the zwitterionic **1** with a carbon acid pK_a of 17. Protonation of **1** at the phenolate oxygen to form **1-H** results in a decrease in the carbon acid pK_a to 11, while its further protonation at the carboxylate anion to form **1-H₂** results in a decrease to a pK_a of 6 [32]. **1-H₂** is an extremely strong carbon acid and would be substantially ionized at pH 7. The very large increases in carbon acidity that are observed as **1** is protonated in solutions of decreasing pH are balanced by the decrease in the concentration of the reactive base hydroxide ion in these solutions so that the *observed* rate constant for deprotonation of total **1** by solvent is nearly pH-independent [32]. There is good agreement between the α -amino carbon acidity of glycine zwitterion determined by experiment [15] and the carbon acidity of alanine obtained from quantum mechanical and molecular mechanical calculations [34,35]. Experiments [32] and calculations [34,35] that examine the effect of formation of iminium ion adducts with pyridoxal on the carbon acidity of amino acids are also in good agreement.
- (2) Formation of an iminium ion adduct with the simple ketone acetone is expected to cause a ca. 7-unit decrease in the pK_a of 29 for deprotonation of the α -amino carbon of glycine zwitterion to a pK_a of ca. 22 [11], which is more than 50% of the 12-unit decrease in this pK_a that is observed upon formation of the **DPL**-glycine iminium ion adduct **1** [32]. The formation of an iminium ion adduct of glycine with phenylglyoxylate results in an even larger

15-unit decrease in the carbon acidity of glycine to a pK_a of 14 [33]. These data show that the **DPL**-glycine iminium ion adduct 1 is not a *uniquely* strong carbon acid. The results of computational studies from the laboratories of Toney [36] and Bach [37] also predict a relatively small role for the pyridine ring of **PLP** in carbanion stabilization. These data are consistent with the generalization that the methyl carbanion is strongly stabilized by the addition of a single resonance electron-withdrawing group, but that there is a sharp falloff in the effects of a second and a third resonance electron-withdrawing group, as a result of the *saturation* of resonance substituent effects [38,39].

Substrate Specificity of PLP Enzymes

We have suggested that **PLP** enzymes select the zwitterionic **PLP**-amino acid iminium complex that is analogous to 1, where the α -imino carbon has the relatively high pK_a of 17 (Figure 5) [32]. There is a large thermodynamic preference for formation of zwitterionic carbanions at relatively nonpolar enzyme active sites [40,41] which provides a strong mechanistic imperative for protonation of the imine nitrogen at the **PLP**-amino acid adduct. Protonation of weakly basic sites at 1 favors deprotonation of the α -imino carbon (Figure 5), but this is balanced by the thermodynamic price paid at neutral pH for these unfavorable proton transfers. Furthermore, enzyme catalysts are expected to show a preference for binding ligands with the maximum number of charged groups, because this maximizes the favorable interactions with amino acid side-chains of opposite charge.

In short, reducing the pK_a of the α -amino carbon of amino acids is one of many functions of **PLP** in enzyme catalysis, but it is not an *indispensable* function. Interactions between the “pure protein” catalyst proline racemase and its substrate proline [42,43] provide the 19 kcal/mol transition state stabilization needed for efficient catalysis of deprotonation of the α -amino carbon [44]. A similar catalytic power is observed for other amino acid racemases/epimerases [45,46]; and, the pyruvoyl prosthetic group also provides a strong stabilization of the carbanion intermediates of decarboxylation reactions [17,47]. If the “intrinsic substrate binding energy” [48] of a *single* amino acid is sufficient to allow for formation of zwitterionic carbanions at racemases, then the total binding energy of the **PLP**-amino acid iminium ion must greatly exceed that needed for catalysis of deprotonation of α -amino carbon. We propose that this “excess” binding energy is one resource drawn upon by the **PLP** in the catalysis of complex enzymatic reactions.

Protonation State of the Pyridine Ring of PLP

While the pyridine ring of **PLP** may not play a critical role in stabilization of α -amino carbanions by enzymes, there is evidence that **PLP** enzymes control the state of its protonation at nitrogen, in order to direct the partitioning of enzyme-bound carbanion intermediates [9]. The X-ray crystal structure of alanine racemase shows that the guanidinium side-chain of Arg-219 is within hydrogen bonding distance of the *neutral pyridine* nitrogen of the **PLP** cofactor [49], while the X-ray crystal structures of D-amino acid transaminase [50,51] and alanine glyoxylate aminotransferase [52] reveal the *protonated pyridinium* nitrogen of **PLP** to be hydrogen-bonded to the carboxylate side-chains of Glu-177 and Asp-180, respectively. Proton transfer from the pyridinium nitrogen of **PLP** ($pK_a = 5$ in water [20]) to the guanidine side-chain of Arg-219 at alanine racemase to form a guanidinium cation ($pK_a = 13$ in water [53]) should be strongly favorable thermodynamically, so that the pyridine ring of **PLP** binds to this enzyme in its basic form (Figure 6, lower section). There should be minimal delocalization of negative charge at the **PLP**-stabilized alanine carbanion onto the *neutral* pyridine ring, which will favor the localization of negative charge at the α -imino carbon (Figure 6, lower section). By comparison, the ca. 11 kcal/mol larger driving force for proton transfer to the pyridine nitrogen from the carboxylic acid side-chain ($pK_a = 5$) at transaminases favors

protonation of the pyridine nitrogen (Figure 6, upper section). This *enables* delocalization of negative charge onto the ring nitrogen and favors the buildup of negative charge at the α -pyridyl carbon. Such strong delocalization of negative charge across the α -imino and α -pyridyl carbons should favor the 1,3-isomerization reaction catalyzed by transaminases.

The preferred reaction pathway for D-amino acid transaminase changes with changing acidity of the amino acid side-chain that is hydrogen bonded to the pyridine/pyridinium nitrogen of **PLP**. The E177K mutation at D-amino acid transaminase leads to a 1,000-fold *decrease* in the specific activity of the enzyme as a transaminase, but a 10-fold *increase* in its racemase activity. This represents a 10,000-fold *increase* in the rate of protonation of the enzyme-bound carbanion at the α -imino carbon relative to the α -pyridyl carbon [50]. The “opposite” R219E mutation at alanine racemase results in a 700-fold decrease in k_{cat}/K_m for racemization of D-alanine, along with the appearance of an absorbance band at 510 nm, consistent with the accumulation of a quinonoid intermediate during turnover [54]. This result shows that there is no simple relationship between the stability of the α -imino carbanion (quinonoid) intermediate and the rate of enzyme-catalyzed racemization.

Binding Energy and Catalysis

An important, but underappreciated, role of the **PLP** cofactor is to provide binding energy that may be utilized for stabilization of the α -imino carbanion (quinonoid) intermediates of a host of **PLP**-dependent enzyme-catalyzed reactions. The phosphodianion groups of D-glyceraldehyde 3-phosphate, orotidine 5'-monophosphate and dihydroxyacetone phosphate each provide a ca. 12 kcal/mol stabilization of the respective transition states for proton transfer catalyzed by triosephosphate isomerase (TIM superfamily) [55], decarboxylation catalyzed by orotidine 5'-monophosphate decarboxylase (OMPDC, ribulose-phosphate binding barrel superfamily) [56], and hydride transfer catalyzed by glycerol 3-phosphate dehydrogenase (GPDH) [57]. Part of this binding energy is expressed at the ground state Michaelis complex, but in all three cases more than 50% of the intrinsic binding energy of the substrate phosphodianion group is expressed specifically at the transition state [56–58].

The **PLP**-dependent enzymes alanine racemase, ornithine decarboxylase and diaminopimelate decarboxylase are members of the **PLP**-binding barrel superfamily, and exhibit a highly conserved phosphate binding motif similar to that of members of the TIM and ribulose-phosphate binding barrel superfamilies [59]. These enzymes are part of a larger group of 24 **PLP**-dependent enzymes that share a common phosphate group recognition pattern [60]. We suggest that interactions of the phosphate binding motif of the **PLP**-binding barrel superfamily members, and possibly of other **PLP**-dependent enzymes, with the phosphodianion group of **PLP** may result in a large transition state stabilization, similar to that of ca. 12 kcal/mol observed for catalysis by TIM, OMPDC and GPDH. This stabilization corresponds to a rate acceleration of ca. 10^8 -fold, which is similar to the estimated 2×10^8 -fold rate acceleration for deprotonation of the α -imino carbon of the **PLP**-bound amino acid effected by alanine racemase [32].

Conclusions

Most of the kinetic barrier to deprotonation of α -carbonyl carbon is due to the thermodynamic barrier to formation of highly unstable enolate reaction intermediates [61]. **PLP** is only one of many aromatic aldehydes that might have evolved to catalyze deprotonation of the α -carbon of amino acids by lowering this thermodynamic barrier. The other functional groups at **PLP** enhance its effectiveness as a cofactor. The phenoxy-like oxygen anion promotes catalysis through formation of an intramolecular hydrogen bond to the iminium nitrogen, which stabilizes the iminium cation relative to glycine zwitterion. The protonation state of the pyridine

nitrogen may influence the outcome of **PLP**-catalyzed reactions. This nitrogen, along with the phosphodianion group of **PLP**, provides substantial intrinsic binding energy that may be harnessed for stabilization of the multiple transition states involved in the many complex reactions catalyzed by **PLP** enzymes [2,28,29,62]. A defining challenge of mechanistic enzymology – the development of an understanding of the mechanism by which enzymes stabilize transition states for their catalyzed reactions – takes on an added level of complexity for **PLP** enzymes that stabilize multiple transition states at different enzyme conformational states [2]. This no doubt requires a substrate binding energy in “excess” of that needed to stabilize a single transition state, and is an imperative for the evolution of highly functionalized cofactors such as **PLP** that form covalent adducts to their substrates.

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Figure 1.

The mechanism for catalysis of deprotonation of glycine methyl ester by the coordinated action of the simple ketone acetone and a Brønsted base B. Electrophilic catalysis by acetone is a result of the 7-unit decrease in the $\text{p}K_{\text{a}}$ of the α -amino carbon upon formation of the iminium ion adduct of glycine methyl ester with acetone [11]. The tertiary amine 3-quinuclidinone (**Q**) acts as a bifunctional catalyst of deprotonation of glycine methyl ester.

Figure 2.

PLP strongly favors heterolytic cleavage of bonds to groups R_1 at the α -amino carbon of amino acids, by providing an “electron sink” to stabilize the negative charge that remains at this carbon after bond cleavage. **PLP** is used as a cofactor in enzyme-catalyzed racemization and transamination ($R_1 = \text{H}$), decarboxylation ($R_1 = \text{CO}_2^-$) and retroaldol cleavage ($R_1 = \text{CH}_2\text{OH}$) reactions. The formal stabilized carbanion intermediate of these reactions is often referred to as a “quinonoid”, which emphasizes the extensive delocalization of negative charge from the α -imino carbon onto the pyridine ring of the **PLP** cofactor.

Figure 3.

(A) Mechanism for the Claisen-type addition of glycine to **DPL** observed in D₂O at neutral pD [22]. This reaction is much faster than **DPL**-catalyzed deuterium exchange between D₂O and the α -amino protons of glycine, because the **DPL**-stabilized glycine carbanion (**DPL=Gly⁻**) reacts with a second molecule of **DPL** much faster than it undergoes protonation by solvent ($k_{\text{add}}[\text{DPL}] \gg k_p$). (B) Condensation of the PLP-stabilized glycine carbanion with succinyl-CoA to form a β -keto acid in the reaction catalyzed by 5-aminolevulinate synthase. The β -keto acid undergoes cofactor-catalyzed decarboxylation at the enzyme active site.

Figure 4.

The mechanism for two additional condensation reactions of **DPL**. **A**. At high pH there is nearly quantitative conversion of **DPL** and glycine to the **DPL**-glycine iminium ion adduct (**DPL=Gly**). This adduct then undergoes addition of the **DPL**-stabilized glycine carbanion **DPL=Gly⁻** to form the novel products of addition of two equivalents of glycine to **DPL** [28]. **B**. The reaction between alanine and **DPL** gives a good yield of the dimeric products of addition of the α -pyridyl carbon of the **DPL**-stabilized alanine carbanion to a second molecule of **DPL** [29].

Figure 5.

The carbon acid pK_a s of glycine zwitterion and the iminium ion adducts that form from addition of glycine to **DPL** [30], acetone, and phenylglyoxylate [31]. The pK_a of 22 for the acetone-glycine iminium ion adduct is estimated with the assumption that iminium ion formation results in the same 7-unit decrease in carbon acid pK_a that we determined for glycine methyl ester [11].

Alanine Racemase

D-Aminoacid Aminotransferase
Alanine Glyoxylate Aminotransferase **Figure 6.**

A comparison of deprotonation of the **PLP**-alanine iminium ion adduct catalyzed by amino acid racemases and transaminases. The pyridine nitrogen of **PLP** is *deprotonated* by the strongly basic guanidine side-chain of Arg-219 at the active site of alanine racemase. The **PLP**-stabilized alanine carbanion is proposed to show minimal delocalization of negative charge onto the pyridine ring nitrogen. This favors charge localization at the α -imino carbon, which is then reprotonated by the racemase. The pyridine nitrogen of **PLP** is *protonated* by the carboxylic acid side-chains at the active sites of D-amino acid aminotransferase and alanine glyoxylate aminotransferase. This promotes delocalization of negative charge from the α -imino carbon of the carbanion intermediate onto the pyridine ring nitrogen. This will increase the negative charge density at the α -pyridyl carbon and protonation of this carbon results in the 1,3-proton shift catalyzed by transaminases.