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# Role of the Pyridine Nitrogen in Pyridoxal 5′-Phosphate Catalysis: Activity of Three Classes of PLP Enzymes Reconstituted with Deazapyridoxal 5′-Phosphate

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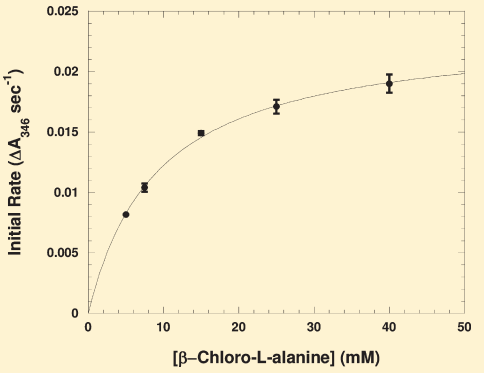
Supporting Information

**ABSTRACT:** Pyridoxal 5′-phosphate (PLP; vitamin $B_6$)-catalyzed reactions have been well studied, both on enzymes and in solution, due to the variety of important reactions this cofactor catalyzes in nitrogen metabolism. Three functional groups are central to PLP catalysis: the C4′ aldehyde, the O3′ phenol, and the N1 pyridine nitrogen. In the literature, the pyridine nitrogen has traditionally been assumed to be protonated in enzyme active sites, with the protonated pyridine ring providing resonance stabilization of carbanionic intermediates. This assumption is certainly correct for some PLP enzymes, but the structures of other active sites are incompatible with protonation of N1, and, consequently, these enzymes are expected to use PLP in the N1-unprotonated form. For example, aspartate aminotransferase protonates the pyridine nitrogen for catalysis of transamination, while both alanine racemase and *O*-acetylserine sulfhydrylase are expected to maintain N1 in the unprotonated, formally neutral state for catalysis of racemization and $\beta$-elimination. Herein, kinetic results for these three enzymes reconstituted with 1-deazapyridoxal 5′-phosphate, an isosteric analogue of PLP lacking the pyridine nitrogen, are compared to those for the PLP enzyme forms. They demonstrate that the pyridine nitrogen is vital to the 1,3-prototropic shift central to transamination, but not to reactions catalyzed by alanine racemase or *O*-acetylserine sulfhydrylase. Not all PLP enzymes require the electrophilicity of a protonated pyridine ring to enable formation of carbanionic intermediates. It is proposed that modulation of cofactor electrophilicity plays a central role in controlling reaction specificity in PLP enzymes.




Within a decade after the isolation and structural characterization of pyridoxine,[1,2] the requirement for pyridoxal 5′-phosphate (PLP) in enzymatic transamination, racemization, and $\beta$-elimination was recognized.[3] The basic structural features essential for enzymatic and nonenzymatic vitamin $B_6$-catalyzed reactions were outlined in 1954 by Metzler et al., who proposed a general mechanism for these reactions.[4] The essential catalytic requirements include an aldehyde at C4′, a phenol at C3′, and the pyridine nitrogen as N1 (see Figure 1 for numbering).

A protonated pyridine nitrogen acting as an electron sink in carbanion stabilization (Figure 1, "quinonoid" resonance form) has since become the paradigm for PLP-catalyzed reactions, invoked in the majority of mechanisms presented in both journals and textbooks.[5−14] Despite the widespread assumption that the pyridine nitrogen must be protonated to form a carbanionic intermediate from the external aldimine, the extent of protonation of N1 in different PLP enzymes, and thereby the electrophilic propensity of PLP, is expected to vary because the acidity of the side-chain functional group interacting with it varies (Figure 1).[14−16] Additionally, recent experimental and computational studies indicate that the protonated Schiff base (Figure 1) plays an important role in carbanion stabilization.[11,17−20] One expects this based on the large body of literature detailing the catalytic proficiency of Schiff bases in various other PLP-independent enzyme mechanisms that entail carbanionic intermediates.[21−23]

Of the five PLP enzyme fold types known, types I, II, and III are of particular interest here because together they constitute the majority of known activities and cover the variations in environment at N1. Aspartate aminotransferase (AAT), a representative fold type I enzyme, can fully protonate N1 ($pK_a$ ∼6 for amino acid aldimines in water) through a salt bridge with Asp222[24] ($pK_a$ ∼4 for the free amino acid).[25] *O*-Acetylserine sulfhydrylase (OASS), a fold type II enzyme, employs Ser272 ($pK_a$ ∼14 for the free amino acid)[26] for hydrogen bonding with N1, ostensibly precluding proton transfer to N1.[27] Likewise, alanine racemase (AR), a fold type III enzyme, places Arg219 ($pK_a$ ∼13 for the free amino acid) within hydrogen-bonding distance of N1 and thus employs PLP in the formally N1-unprotonated form.[15]

Previous experiments aimed at delineating the role of N1 in PLP enzymes relied on either nonisosteric coenzyme analogues or mutation of amino acid residues interacting with the pyridine ring. For example, the D222A mutant of AAT[28] and the R219A mutant of AR[29] have $k_{cat}$ values decreased by $10^5$- and $10^3$-fold,

**Received:** July 1, 2011
**Published:** August 09, 2011





dx.doi.org/10.1021/ja2061006 | *J. Am. Chem. Soc.* 2011, 133, 14823–14830

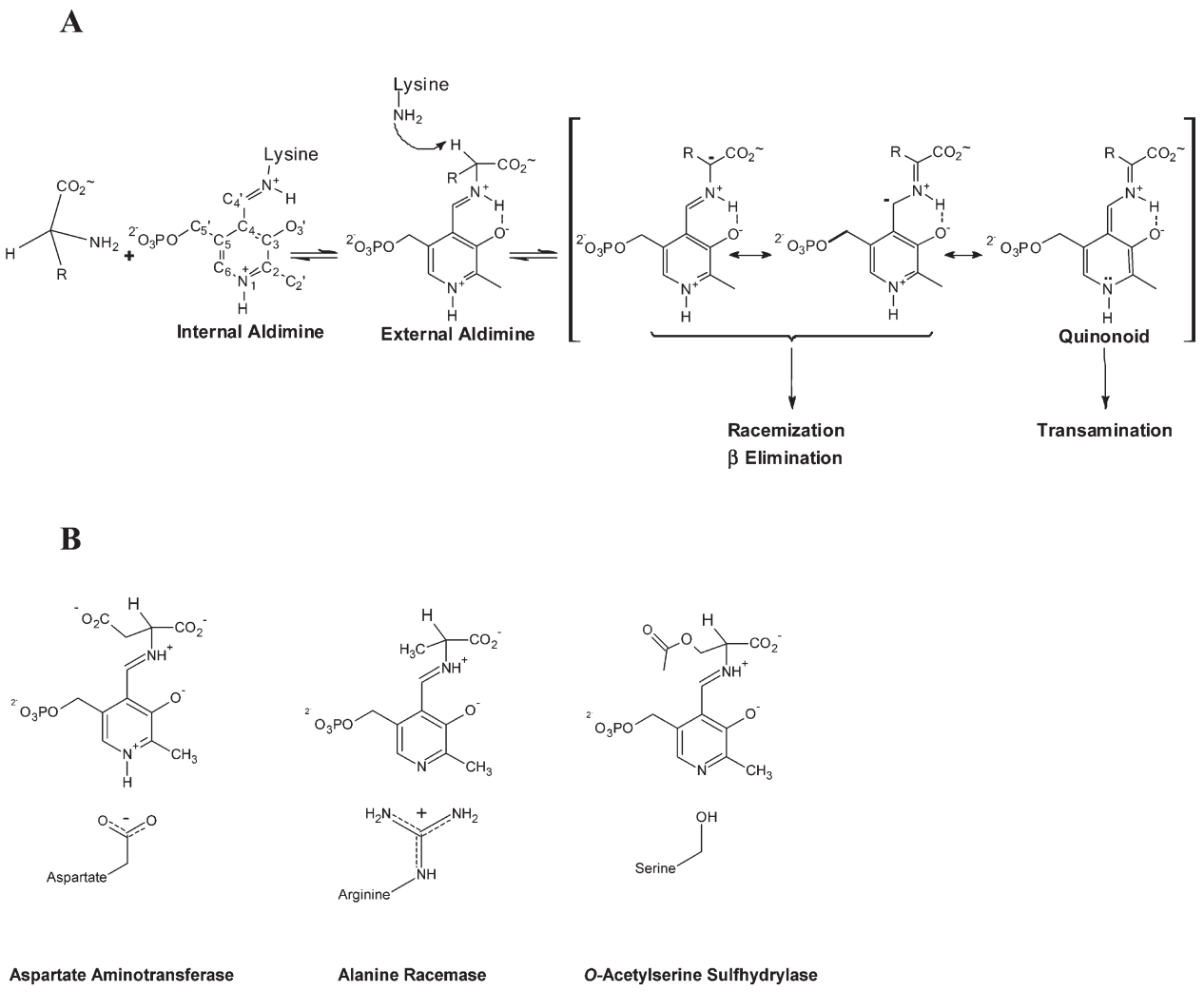



**Figure 1.** (A) Aldimine intermediates and resonance forms of the carbanionic intermediate in PLP-dependent reactions. The external aldimine intermediate is common to all PLP-catalyzed reactions. (B) The protonation state of the pyridine nitrogen (N1) is expected to vary with the acidity of the amino acid residue interacting with it in PLP-dependent enzymes. Aspartate aminotransferase fully protonates N1 via Asp222. Alanine racemase and *O*-acetylserine sulfhydrylase likely maintain N1 in a formally neutral state through interactions with Arg219 and Ser272, respectively.

respectively, while $k_{cat}/K_m$ for the first half-reaction of the S272A mutant of OASS is decreased by only ~4-fold.[30] We recently reported the synthesis of an isosteric, carbocyclic analogue of PLP, 1-deazapyridoxal 5′-phosphate (deazaPLP, Figure 2).[31] DeazaPLP, possessing nether the potential for protonation nor the greater electronegativity of nitrogen versus carbon, is expected to be a poor substitute for PLP with AAT, but have greater activity for AR and OASS, which apparently employ PLP in an N1-unprotonated state. Results presented herein confirm this prediction.

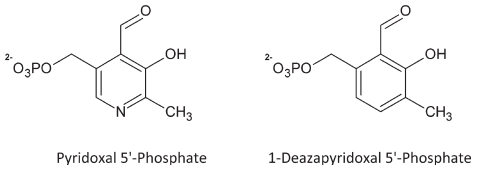



**Figure 2.** Structures of PLP and its carbocyclic analogue, deazaPLP.

## EXPERIMENTAL SECTION

**Materials.** L-Aspartic acid, L-glutamic and L-cysteine sulfinic acid (L-cysteine sulfinate), β-chloro-L-alanine, *O*-acetyl-L-serine (OAS), HEPES, triethanolamine (TEA), and the reduced form of nicotinamide adenine dinucleotide (NADH) were from Sigma-Aldrich. Hydroxylamine hydrochloride, ammonium sulfate, and guanidine hydrochloride were from Fisher. Lactate dehydrogenase (LDH) was from Calbiochem. DeazaPLP was synthesized as described.[31]

**Enzymes.** *1. AAT.* Enzyme was prepared as previously described.[32] Apoenzyme preparation also followed a literature procedure.[33] It was treated with 10 mM sodium borohydride in 100 mM TEA, pH 8.0, for 20 min at room temperature followed by dialysis.

*2. AR.* The wild-type enzyme from *Geobacillus stearothermophilus* was expressed and purified as described.[29] The apoenzyme was obtained according to literature methods[34] and treated with 10 mM sodium borohydride in 100 mM TEA, pH 8.0, for 20 min at room temperature and then dialyzed against two changes of 100 mM TEA, pH 8.0. This was followed by treatment with 50 mM β-chloroalanine in 100 mM TEA, pH 7.5, for 30 min at 4 °C.

*3. OASS.* [35]Protein expression and purification of the wild type enzyme from *Salmonella typhimurium* was carried out as described.[36]

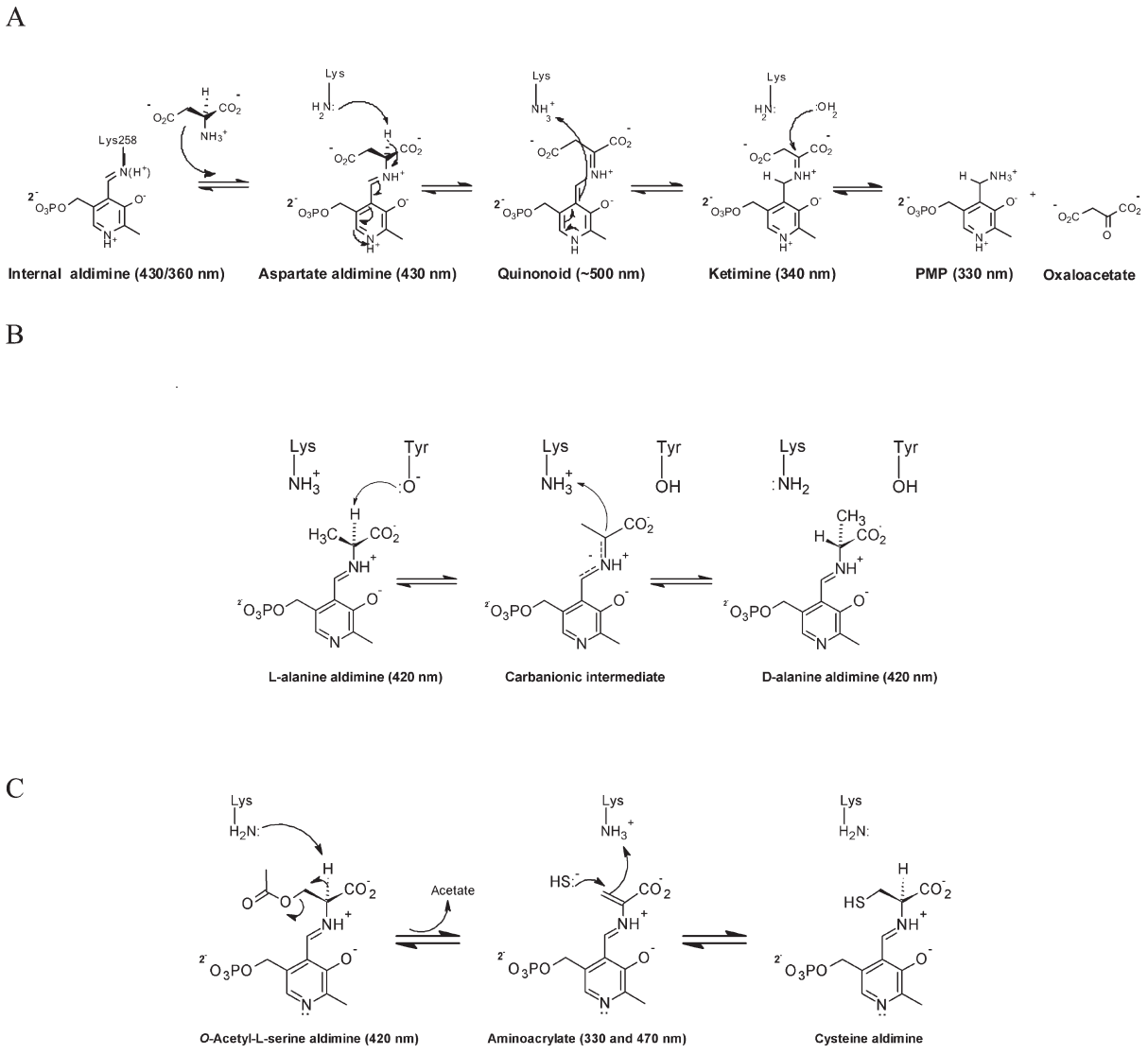



**Figure 3.** (A) Mechanism of the AAT transamination half-reaction with L-aspartate. (B) Mechanism of the AR-catalyzed racemization of alanine. (C) Mechanism of the OASS catalyzed formation of L-cysteine from *O*-acetyl-L-serine and sulfide.

The apoenzyme was prepared according to literature methods[37] and treated with 10 mM sodium borohydride in 100 mM TEA, pH 8.0, for 20 min at room temperature.

Reconstitution of all three apoenzymes with deazaPLP was accomplished by overnight incubation at 4 °C in the presence of a 5−10-fold molar excess of cofactor over active sites, followed by dialysis against three changes of buffer (AAT, 100 mM TEA-HCl, pH 8.0; OASS, 100 mM HEPES, pH 7.0; AR, 100 mM HEPES, pH 7.2) to a final free deazaPLP concentration of ~5 $\mu$M. UV−vis scans from 250 to 600 nm were taken of the apo, deazaPLP, and PLP forms of all three enzymes to assess the extent of reconstitution.

**Instruments.** UV−vis scans and single wavelength kinetics were measured on a Shimadzu UV-2450 spectrophotometer. Spectra versus time were collected on an Agilent 8453 diode array spectrophotometer. Stopped-flow experiments were performed on an Applied Photophysics SX17.MV stopped-flow spectrophotometer using either a diode-array detector for collecting absorbance spectra or a photomultiplier for single-wavelength monitoring.

**Equations.** Steady-state saturation curves were fitted to the Michaelis−Menten equation (eq 1). Single-turnover saturation curves were fitted to eq 2. $\Delta\Delta G^{\ddagger}$ values were calculated using the Erying equation (eq 3).

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$k_{obs} = \frac{k_1[S]}{K + [S]} \quad (2)$$

$$\Delta\Delta G^{\ddagger} = RT \ln\left[\frac{k_1}{k_2}\right] \quad (3)$$

*AAT.* All transamination half-reactions were monitored by the disappearance of the external aldimine peak (~430 nm for AAT/PLP and ~420 nm for AAT/deazaPLP, Figure 3). Final concentrations for all reactions were 100 mM substrate, 10−20 $\mu$M enzyme, and 100 mM TEA-HCl, pH 8.0. To render the reaction of AAT/PLP with L-cysteine sulfinate irreversible, morpholine (600 mM final concentration) was


dx.doi.org/10.1021/ja2061006 |*J. Am. Chem. Soc.* 2011, 133, 14823–14830

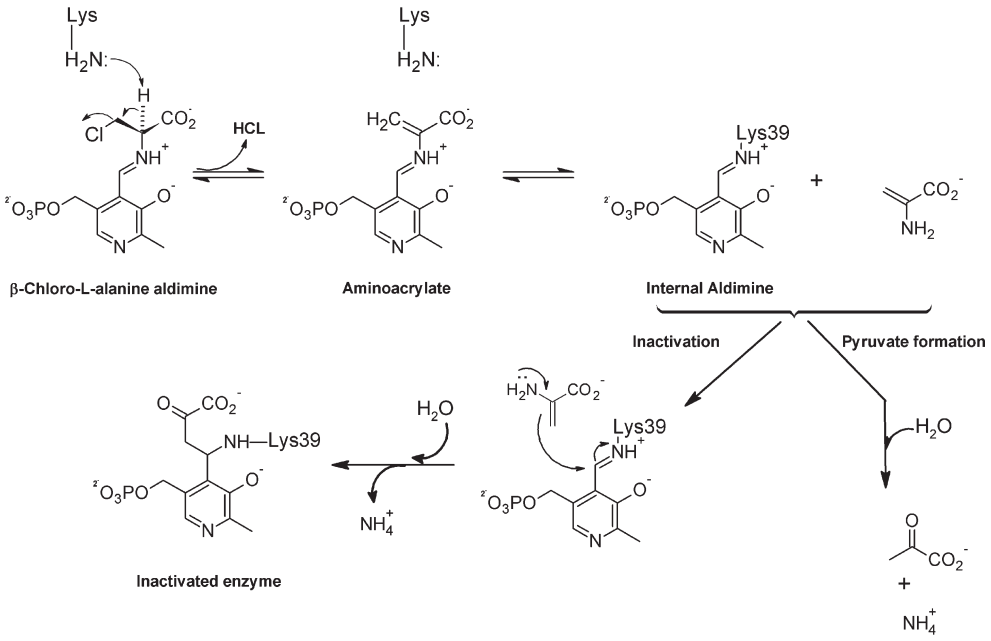



**Figure 4.** Mechanism of the reaction of AR with $\beta$-chloroalanine showing both pyruvate formation and inactivation of the enzyme.

**Table 1. Kinetic Parameters for PLP and deazaPLP Forms of AAT**

| substrate | AAT/PLP $k_{max}$ ($s^{-1}$) | AAT/deazaPLP $k_{max}$ ($s^{-1}$) | $k_{max}$ ratio[a] | $\Delta\Delta G^{\ddagger}$[b] (kJ/mol) |
|---|---|---|---|---|
| L-cysteine sulfinate | 1.5 (0.1) × $10^{3}$ | 5.1 (0.1) × $10^{-6}$ | 2.9 (0.3) × $10^{8}$ | 48 (5) |
| L-aspartate | 5.3 (0.3) × $10^{2}$[c] | ND[d] | >$10^{9}$ | |
| L-glutamate | 7 (1) × $10^{2}$[c] | ND[d] | >$10^{9}$ | |

[a] $k_{max}$ for the PLP enzyme divided by $k_{max}$ for the deazaPLP enzyme. [b] Calculated from eq 3. [c] Values from ref 21. [d] No reaction detected. Errors are given in parentheses.

**Table 2. Kinetic Parameters for the PLP and deazaPLP Forms of Alanine Racemase**

| | AR/PLP | | AR/deazaPLP | | | |
|---|---|---|---|---|---|---|
| substrate | $k_{cat}$ ($s^{-1}$)[a] | $K_m$ (mM)[a] | $k_{cat}$ ($s^{-1}$)[a] | $K_m$ (mM)[a] | $k_{cat}$ ratio[b] | $\Delta\Delta G^{\ddagger}$[c] (kJ/mol) |
| $\beta$-chloro-L-alanine | 8.5 (0.5)[a] | 9.2 (0.5)[a] | 3.1 (0.1) × $10^{-2}$[a] | 102 (3)[a] | 2.6 (0.2) × $10^{2}$ | 14 (1) |
| L-alanine | 1.4 (0.1) × $10^{3}$[d] | 3.0 (0.3)[d] | <2 | | >700 | >16 |

[a] Calculated from eq 1. [b] $k_{cat}$ for the PLP enzyme divided by $k_{cat}$ for the deazaPLP enzyme. [c] Calculated from eq 3. [d] Values from ref 22. Errors are given in parentheses.

added to catalyze the decomposition of the product $\beta$-sulfinylpyruvate to pyruvate and bisulfite.[38] The AAT/PLP reaction with L-cysteine sulfinate was monitored with a stopped-flow spectrophotometer, collecting 1000 data points in 10 ms. Data were fit to a single exponential equation using software provided with the instrument. The AAT/deazaPLP reactions with L-aspartate, L-glutamate, and L-cysteine sulfinate were monitored over 50 h with scans collected every 30 min from 250 to 700 nm, with the average absorbance between 650 and 700 nm subtracted from each reading to correct for baseline drift. Global fitting of the spectra (300−600 nm) to a single exponential equation was performed with SPECFIT (Spectrum Software Associates).

*AR.* Racemization of D- to L-alanine (Figure 3) was followed as previously described.[29] Pyruvate production from the reaction of AR with $\beta$-chloroalanine[39−41] (Figure 4) was monitored with LDH-catalyzed NADH oxidation.[41] AR/PLP and AR/deazaPLP reactions contained (final concentrations) 100 mM HEPES, pH 7.2, 200 $\mu$M NADH, 25 units/mL LDH, and varying concentrations of $\beta$-chloroalanine. Reactions were initiated by addition of AR/deazaPLP to 10 $\mu$M or by rapid mixing of a 1.0 $\mu$M AR/PLP solution in the stopped-flow to give a final protein concentration of 0.5 $\mu$M. The rapid deactivation (half-life ~10 s) of wild-type AR in the presence of $\beta$-chloroalanine is accompanied by a decrease in the absorbance at ~420 nm, and an increase at ~330 nm with an isosbestic point at 346 nm (Figure 6). The molar absorptivity of NADH at 346 nm was determined to be 5884 $M^{-1}$ $cm^{-1}$, and the AR/PLP reactions were monitored at this wavelength with a stopped-flow spectrophotometer, collecting 4000 points over 5 s. The initial linear portion of the data (from 1.5 to 3 s) was used to calculate an initial rate. Over a period of 20 h, the UV−vis spectra of AR/deazaPLP in the presence of 50 mM $\beta$-chloroalanine in 100 mM HEPES, pH 7.2, showed no decrease in absorbance at ~410 nm, indicating no enzyme inactivation. This permitted the molar absorptivity for NADH of 6220 $M^{-1}$ $cm^{-1}$ at 340 nm to be used. Initial rates were fitted to eq 1 for both the PLP and the deazaPLP enzyme forms.

**Table 3. Kinetic Parameters for the PLP and deazaPLP Forms of *O*-Acetylserine Sulfhydrylase**

| | OASS/PLP | | OASS/deazaPLP | | | |
|---|---|---|---|---|---|---|
| substrate | $k_{max}$ ($s^{-1}$)[a] | $K_{app}$ (mM)[a] | $k_{max}$ ($s^{-1}$)[a] | $K_{app}$ (mM)[a] | $k_{max}$ ratio[c] | $\Delta\Delta G^{\ddagger}$[d](kJ/mol) |
| *O*-acetyl-L-serine | 800 (30) | 1.4 (0.2) | 3.1 (0.2) | 0.6 (0.1) | 260 (30) | 14 (2) |

[a] Calculated from eq 2. [c] $k_{max}$ for the PLP enzyme divided by $k_{max}$ for the deazaPLP enzyme. [d] Calculated from eq 3. Errors are given in parentheses.

*OASS.* Aminoacrylate formation was monitored as described (Figure 3).[36] Final concentrations for the reactions of the PLP and deazaPLP forms of OASS were 100 mM HEPES, pH 7.0, varying concentrations of OAS, and 10−15 $\mu$M enzyme. The OASS/PLP reaction was monitored 467 nm with the stopped-flow, collecting 1000 data points over 10 ms. Data were fitted to a single exponential equation. Time-dependent absorbance scans from 250 to 600 nm for the OASS/deazaPLP reaction were obtained with the stopped-flow. Spectral data were globally fitted to a single exponential with SPECFIT. The observed rate constants for aminoacrylate formation versus OAS concentration for the PLP and deazaPLP form of OASS were fitted to eq 2. All reactions were carried out at 25 °C and repeated at least in triplicate.

## RESULTS

Tables 1, 2, and 3 summarize kinetic parameters and $\Delta\Delta G^{\ddagger}$ values for the PLP and deazaPLP forms of AAT, AR, and OASS, respectively.

**AAT.** The internal aldimine of AAT/deazaPLP exhibits maximal absorbance at ~420 nm, compared to that of AAT/PLP at ~430 nm (protonated internal aldimine) and ~360 nm (unprotonated internal aldimine) (Figure 5A). No discernible reaction (i.e., decrease in 420 nm absorbance) between 100 mM L-aspartate or L-glutamate and AAT/deazaPLP was observed over 50 h. The half-life of these reactions is therefore estimated to be >20 days, corresponding to a $>10^9$-fold decrease in reactivity compared to AAT/PLP. Figure 5B shows spectra taken over a 50 h period for AAT/deazaPLP reacting with 100 mM L-cysteine sulfinate. The observed rate of AAT/deazaPLP-catalyzed transamination of L-cysteine sulfinate represents a $\sim10^8$-fold decrease in activity compared to the AAT/PLP-catalyzed reaction.

**AR.** The maximal absorbance of AR/deazaPLP is at ~410 nm, compared to ~420 nm for the AR/PLP internal aldimine (Figure 6B). Treatment of the apoenzyme with sodium borohydride and $\beta$-chloroalanine gave apoAR preparations that still retained ~2% residual racemization activity. This was reproducible over multiple apoAR preparations. No alanine racemization due to AR/deazaPLP was detectable over this apoAR background activity.

Unlike the AAT and OASS reactions, the absence of UV−vis absorbance changes associated with AR-catalyzed racemization precludes direct spectral monitoring of reactions (Figure 3). AR is known to catalyze the elimination of HCl from $\beta$-chloroalanine, which requires Cα−H deprotonation as the first step, and additionally forms pyruvate and ammonia (Figure 4).[39−41] As previously demonstrated, $\beta$-chloroalanine also inactivates AR/PLP with a half-life of ~10 s, leading to a decrease in absorbance at 420 nm (Figure 6A). In contrast, AR/deazaPLP shows no decrease in the ~410 nm peak over a 20 h period, indicating no inactivation (Figure 6B). This allowed $k_{cat}$ for the elimination of HCl and subsequent pyruvate formation from $\beta$-chloroalanine catalyzed by AR/deazaPLP to be determined (Table 2). The ~275-fold decrease in $k_{cat}$ for AR/deazaPLP compared to AR/PLP corresponds to $\Delta\Delta G^{\ddagger}$ of ~14 kJ/mol.

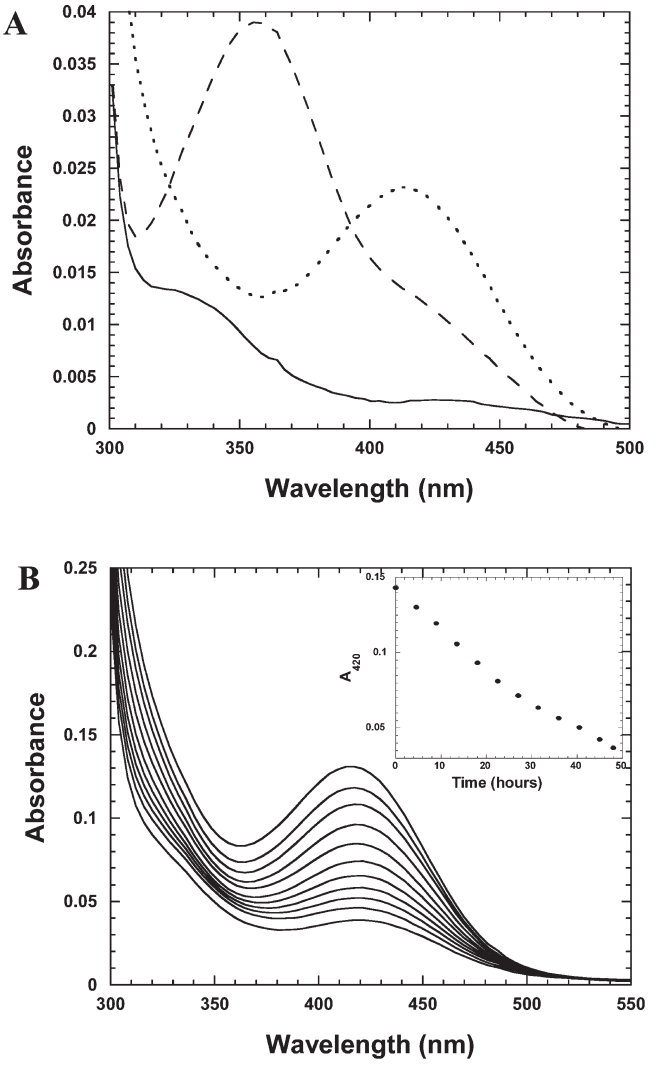



**Figure 5.** (A) UV−vis spectra of apoAAT (−), AAT/PLP (− − −), and AAT/deazaPLP (· · ·). Conditions: 100 mM TEA, pH 8.0, and ~5 $\mu$M enzyme. (B) UV−vis scans taken over 50 h showing the decrease in absorbance at ~420 nm associated with the transamination half-reaction of L-cysteine sulfinate with AAT/deazaPLP. Conditions: 100 mM TEA, pH 8.0, 100 mM L-cysteine sulfinate, and ~25 $\mu$M enzyme.

**OASS.** Reconstitution of apoenzyme with deazaPLP gives an internal aldimine with maximal absorbance at ~400 nm, compared to 412 nm for OASS/PLP (Figure 7A). The overall reaction catalyzed by OASS is comprised of the elimination half-reaction with OAS, followed by the addition half-reaction between the aminoacrylate intermediate and bisulfide. It was previously shown that the elimination half-reaction with OAS limits the overall catalytic cycle.[42] Therefore, the effect of deazaPLP on acetate elimination was studied.

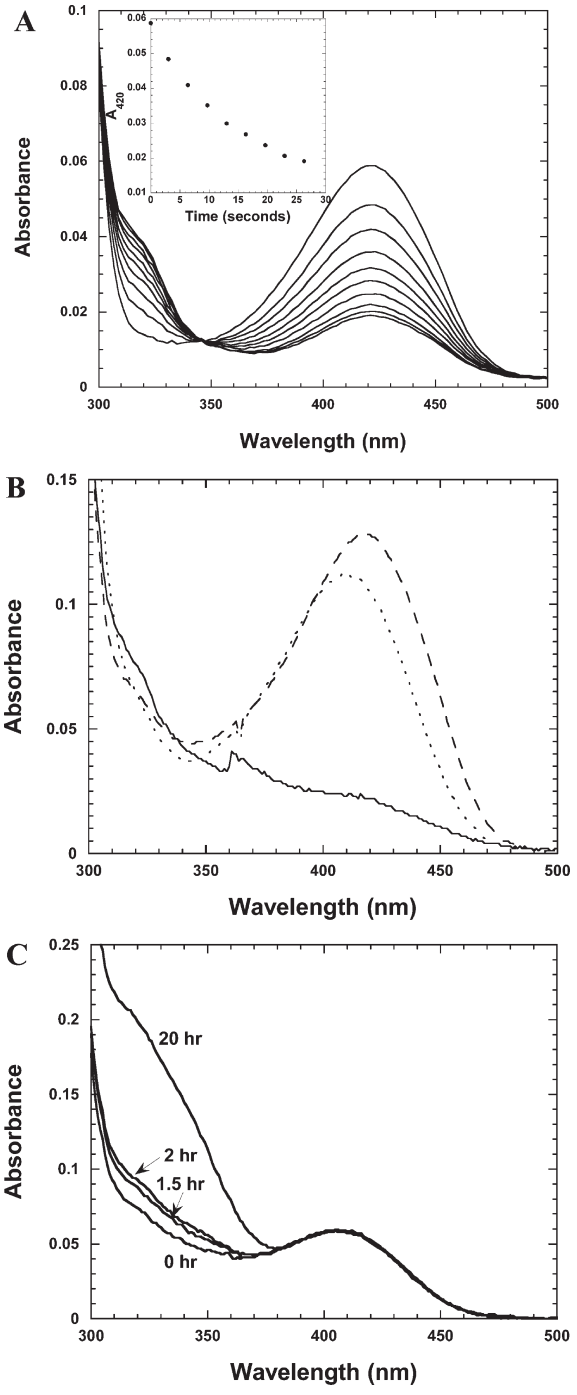



**Figure 6.** (A) Time-dependent UV−vis spectra, collected using a stopped-flow spectrophotometer, showing inactivation of AR/PLP in the presence of 50 mM $\beta$-chloroalanine. There is an isosbestic point at 346 nm. The initial peak at ∼420 nm decreases over time. Conditions: 10 mM $\beta$-chloroalanine, 100 mM HEPES, pH 7.2, and ∼10 $\mu$M AR (final concentrations). (B) UV−vis spectra of apoAR (−), AR/PLP (− − −), and AR/deazaPLP (· · ·). Conditions: 100 mM HEPES, pH 7.2, and ∼15 $\mu$M enzyme. (C) UV−vis scans taken at 0, 1.5, 2, and 20 h showing the stability of AR/deazaPLP in the presence of $\beta$-chloroalanine. The increase in absorbance in the 300−350 nm region is due to pyruvate formation from the $\beta$-elimination of HCl catalyzed by AR/deazaPLP. Conditions: 100 mM HEPES, pH 7.2, 50 mM $\beta$-chloroalanine, and ∼10 $\mu$M enzyme.

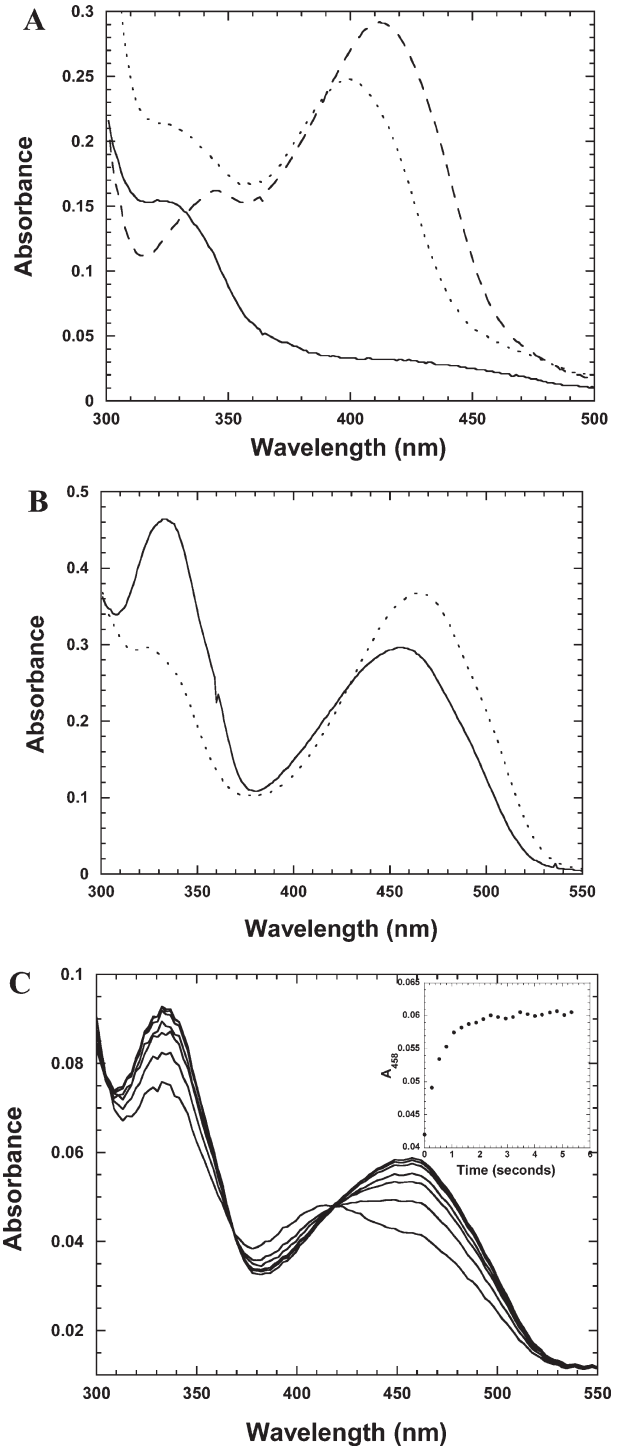



**Figure 7.** (A) UV−vis spectra of apoOASS (−), OASS/PLP (− − −), and OASS/deazaPLP (· · ·). Conditions: 100 mM HEPES, pH 7.0, and ∼35 $\mu$M enzyme. (B) UV−vis spectra of the stable aminoacrylate formed by addition of OAS to OASS/PLP (· · ·) and OASS/deazaPLP (−). Conditions: 100 mM HEPES, pH 7.0, 1 mM OAS, and ∼35 $\mu$M enzyme. (C) Time-dependent UV−vis scans of aminocrylate formation on mixing OAS with OASS/deazaPLP. The inset shows the absorbance changes at 458 nm with time. Conditions: 100 mM HEPES, pH 7.0, 1 mM OAS, and ∼10 $\mu$M enzyme.

On addition of OAS to OASS/deazaPLP, a stable aminoacrylate forms, converting the 400 nm internal aldimine peak to

peaks at 458 and 330 nm. The corresponding OASS/PLP reaction with OAS gives aminoacrylate absorbance at 470 and 330 nm (Figure 7B). These spectral changes allowed direct monitoring of aminoacrylate formation, the rate limiting half-reaction of OASS.[43] Figure 7C shows representative spectral changes for OASS/deazaPLP in the presence of 1 mM OAS, monitored by diode-array detected stopped-flow. The $k_{max}$ for OASS/deazaPLP aminoacrylate formation is ~250-fold less than that for the OASS/PLP reaction, corresponding to $\Delta\Delta G^{\ddagger}$ of ~14 kJ/mol.

## DISCUSSION

The $\sim 10^8$-fold difference in activity ($\Delta\Delta G^{\ddagger}$ = 48 kJ/mol) between AAT/PLP and AAT/deazaPLP in the transamination of L-cysteine sulfinate is striking (Table 1). The reaction of AAT/deazaPLP with L-aspartate is even slower, with a $>10^9$-fold decrease in reactivity compared to AAT/PLP (Table 1). Active site overlays of the AAT/deazaPLP L-aspartate external aldimine (PDB entry 3QN6) with the AAT/PLP α-methyl-L-asparate external aldimine (PDB entry 1ART) show very high structural similarity, indicating that the inactivity of AAT/deazaPLP is not due to disadvantageous binding of deazaPLP.[44] The manifest conclusion from these structures is that the simple change from nitrogen to carbon in the ring causes the drastic loss of catalytic activity. By comparison, the K258A mutation in AAT imparts a $\sim 10^8$-fold decrease in reactivity with L-aspartate.[33] Thus, the presence of nitrogen in the ring is more important to AAT catalysis than is Lys258, the general base catalyst for the central 1,3-prototropic shift (Figure 3A).

One can estimate a $k_{cat}$ value for alanine racemization by AR/deazaPLP as $<2\ s^{-1}$ (>700-fold reduced from AR/PLP) based on the residual activity of $\sim 20\ s^{-1}$ of the apoAR preparation and a limit of detection of 10% above the background rate. It is certainly likely that AR/deazaPLP catalyzes racemization of alanine because nonenzymatic racemization of amino acids by salicylaldehyde derivatives is documented in the literature.[45,46] Unfortunately, the background racemization activity of the apoAR preparations obtained could not be reduced enough to detect the AR/deazaPLP racemization activity, despite extensive efforts to reduce it further.

Both β-elimination reactions analyzed here, AR/deazaPLP with β-chloroalanine and OASS/deazaPLP with OAS, show similar ~250-fold decreases in activity (Tables 2 and 3). Unlike for transamination, the presence of nitrogen in the ring is not essential for good β-elimination activity. Racemization and β-elimination both entail proton transfers at Cα only, but unlike racemization, β-elimination can occur through a concerted mechanism (E2-type) that does not require the formation of a distinct carbanionic intermediate. This may be the source of the larger deleterious effect of deazaPLP on racemization (>700-fold) versus β-elimination (250-fold) catalyzed by AR. Previously, Cook et al. proposed that OASS/PLP indeed catalyzes β-elimination through an E2-type mechanism, which would be even more favored with OASS/deazaPLP.[27]

Previous results with mutant enzymes in which residues interacting with the pyridine nitrogen are truncated, as well as the results presented here with deazaPLP, allow one to separate nominally the effects of deprotonation of N1 and replacement of N1 with carbon. Deprotonation of N1 in AAT (D222A mutant) decreases transamination reactivity by $\sim 10^5$-fold,[28] while replacement of the deprotonated nitrogen with carbon further decreases reactivity by $\sim 10^4$-fold. Thus, the greater electronegativity of nitrogen versus carbon plays an important role in AAT-catalyzed transamination. Results with AR (R219A mutant) show the catalytic advantage of arginine hydrogen bonding to N1 is $10^3$-fold.[29] Replacement of N1 with carbon further reduces reactivity by at least 700-fold for alanine racemization, but only 250-fold for HCl elimination from β-chloroalanine. With OASS, loss of the hydrogen bond with serine (S272A mutant) gives only ~4-fold lower activity than that of wild-type OASS, while replacement of N1 with carbon further reduces reactivity by ~250-fold, indicating that the S272 hydrogen bond with the pyridine nitrogen is less important to OASS catalysis than is the electronegativity of the nitrogen. Thus, AR-catalyzed racemization, with a distinct carbanionic intermediate,[47] has lower electrophilicity requirements than the 1,3-prototropic shift in AAT-catalyzed transamination, but greater electrophilicity requirements than OASS-catalyzed elimination of acetic acid from OAS.

The above results imply that PLP enzymes can at least partially control the fate of the carbanionic intermediate by modulating the charge on the pyridine nitrogen. Recent computational studies show that N1 protonation facilitates protonation of the carbanionic intermediate at C4′ as compared to Cα.[20] In AR, multiple kinetic isotope effects were required to establish the existence of a carbanionic intermediate in racemization of alanine because it was not populated enough to detect directly by UV−vis spectroscopy.[47] The instability of this intermediate in AR was proposed to be responsible for the high ($\sim 10^6$-fold) fidelity for racemization over transamination.[11] Unlike the former, the latter reaction requires rate-limiting movement of the active site lysine or tyrosine to protonate C4′ of PLP, which would be suppressed by the short lifetime of the carbanionic intermediate.[11] An analogous result is obtained with D-amino acid aminotransferase: in the E177K mutant (Glu177 protonates N1 in the wild type), transamination is decreased by 1000-fold, while racemization is increased 10-fold.[18]

The arguments presented above are in accord with chemical precedent.[48] Recent experimental work on carbonyl-catalyzed deprotonation of Cα−H of glycine shows that formation of the N1-unprotonated Schiff base from 5′-deoxypyridoxal and glycine lowers the $pK_a$ of the Cα−H of glycine from ~29 (free amino acid) to ~23. On the other hand, the N1-protonated Schiff base from 5′-deoxypyridoxal and glycine has a lower $pK_a$ of ~17. Crugeiras et al. argue that the greater acidity of the Cα−H of glycine in the N1-protonated Schiff base provides direct evidence for greater delocalization of negative charge within the π-electron system, with higher electron density at C4′ favoring its protonation and thereby the 1,3-prototropic shift central to transamination.[48]

The conventionally accepted role of the pyridine nitrogen in PLP, acting equally in all reactions as an electron sink for carbanion stabilization, must be revised. The results presented herein conclusively demonstrate that not all PLP enzymes require a protonated pyridine nitrogen (or even a pyridine nitrogen at all) to catalyze efficiently their cognate reactions. The role of pyridine nitrogen protonation in enzymes should be expanded to include controlling reaction specificity. By controlling the extent of protonation of the pyridine nitrogen, enzyme active sites can modulate the electrophilic strength of PLP to meet the requirements of the desired reaction while minimizing unwanted side reactions.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Plots of the initial rates of pyruvate formation from $\beta$-chloroalanine catalyzed by AR/PLP and AR/deazaPLP fitted to eq 1 and plots of $k_{obs}$ versus OAS concentration for the PLP and deazaPLP forms of OASS fitted to eq 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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