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Metal Ion Inhibition of Nonenzymatic Pyridoxal Phosphate Catalyzed Decarboxylation and Transamination

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Abstract: Nonenzymatic pyridoxal phosphate (PLP) catalyzed decarboxylations and transaminations have been revisited experimentally. Metal ions are known to catalyze a variety of PLP-dependent reactions in solution, including transamination. It is demonstrated here that the rate accelerations previously observed are due solely to enhancement of Schiff base formation under subsaturating conditions. A variety of metal ions were tested for their effects on the reactivity of the 2-methyl-2-aminomalonate Schiff bases. All were found to have either no effect or a small inhibitory one. The effects of Al^{3+} were studied in detail with the Schiff bases of 2-methyl-2-aminomalonate, 2-aminoisobutyrate, alanine, and ethylamine. The decarboxylation of 2-methyl-2-aminomalonate is unaffected by metalation with Al^{3+}, while the decarboxylation of 2-aminoisobutyrate is inhibited 125-fold. The transamination reaction of ethylamine is 75-fold slower than that of alanine. Ethylamine transamination is inhibited 4-fold by Al^{3+} metalation, while alanine transamination is inhibited only 1.3-fold. Metal ion inhibition of Schiff base reactivity suggests a simple explanation for the lack of known PLP dependent enzymes that make direct mechanistic use of metal ions. A comparison of enzyme catalyzed, PLP catalyzed, and uncatalyzed reactions shows that PLP dependent decarboxylases are among the best known biological rate enhancers: decarboxylation occurs 10^{18}-fold faster on the enzyme surface than it does free in solution. PLP itself provides the lion's share of the catalytic efficiency of the holoenzyme: at pH 8, free PLP catalyzes 2-aminoisobutyrate decarboxylation by $\sim 10^{10}$-fold, with the enzyme contributing an additional $\sim 10^{8}$-fold.

Pyridoxal phosphate (PLP[1]) plays a central role in the amino acid and amine metabolism of all organisms. It is covalently bound at the active site of a very large number of enzymes which effect a variety of types of chemical transformations including decarboxylation, transamination, racemization, $\alpha-\beta$ and $\beta-\gamma$ eliminations, and retro-aldol cleavages. The early work of Snell's group[2−62] on the pure coenzyme and its analogues provided a mechanistic foundation for understanding the enzymatic activities.

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(1) Abbreviations used: PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; MAM, 2-methyl-2-aminomalonate; AIB, α-aminoisobutyrate; TEA, triethanolamine; CHES, 2-[*N*-cyclohexylamino]ethanesulfonic acid; NMR, nuclear magnetic resonance spectroscopy. The following metal salts at millimolar concentrations had no effect on the rate of MAM decarboxylation: $CaCl_2$, $MgCl_2$, CsCl, $CdCl_2$, $MnCl_2$, $NiCl_2$, $CoCl_2$, $Cu(OAc)_2$, $Pb(NO_3)_2$, $Cr(ClO_4)_3$, $Yt(NO_3)_3$, and $TlCl_3$. The decarboxylation rate constant decreased linearly with concentration for the following metal salts: $Sm(OAc)_3$, $ErCl_3$, $EuCl_3$, and $La(NO_3)_3$.

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It was observed in these early studies that metal ions promote many of the nonenzymatic reactions.[7,8] A wide variety of di- and trivalent metal ions are active. The Al^{3+} ion is a well-studied example and is a particularly appropriate comparison to the proton because of its small ionic radius. The early studies cited above were semiquantitative at best in their analyses. No attempt was made to determine the relative magnitudes of the effects of the metal ions on promotion of Schiff base formation and on the reactivities of the metal ion bound Schiff bases.

The assumption in the literature has been that the multiple charge of the Schiff base bound metal ion is more effective than the unitary charge of a proton at stabilizing the carbanion formed on heterolytic cleavage of a bond to Cα of the amino acid.[6] Extensive studies from Bruice's laboratory (ref 9 and references therein) demonstrating and characterizing reactions in the absence of metal ions showed their ancillary nature in nonenzymatic systems. The absence in the literature of reports of PLP dependent enzymes that make direct mechanistic use of metal ions additionally suggests that they provide no fundamental advantage to catalysis. This paper describes experiments in which the reactivities of four Schiff bases of amino acids and amines are examined in the presence and absence of Al^{3+}. It is shown that, in all cases, the protonated Schiff base is at least as or more reactive in decarboxylation or transamination compared to the metalated Schiff base.

The rate constants reported herein for decarboxylation of AIB and transamination of alanine and ethylamine in the absence of metal ions allow the calculation of the catalytic ratios for the nonenzymatic and enzymatic PLP dependent reactions. These calculations provide a measure of the catalytic prowess of PLP dependent enzymes relative to others that have been characterized.

Experimental Procedures

Materials. Methylaminomalonic acid was synthesized as previously described[10,11] and stored at −20 °C. D,L-Alanine and PLP were purchased from Sigma. TEA and zinc acetate were obtained from Fisher. Ethylamine was obtained as a 70% aqueous solution from Sigma. Aluminum chloride was from Mallinckrodt. Gallium chloride and AIB were purchased from Aldrich. All references to metal ion concentrations refer to the total metal ion concentration. MAM and PLP solutions were prepared immediately before use, and the PLP solutions were kept in the dark to prevent decomposition.

Reaction Conditions. Reactions contained 0.1 mM PLP, 0.2 M KCl, and 0.2 M potassium acetate (pH 5.0), 0.2 M TEA (pH 8.0), or 0.2 M CHES (pH 9.0). They were maintained at 25 °C in the dark in quartz cuvettes, which were covered with Teflon caps. Reactions that took longer than 2 h to complete were additionally sealed with high-vacuum grease to prevent evaporation. Controls with PLP in the absence of amine were maintained identically to the reaction mixtures. These controls demonstrated that evaporation over the long reaction times is insignificant and that PLP is not degraded under the experimental conditions.

pH Jump. Schiff bases were preformed at pH 8 in 10 mM TEA−HCl buffer, followed by addition of the metal ion in 0.2 M potassium acetate buffer at low pH to bring the solution to the desired pH. This pH jump was done to maximize Schiff base formation using high pH and to use low pH to maximize the rate of metal ion binding and metal ion solubility.

Instruments. Absorbance spectra were collected from 250 to 600 nm with a Hewlett-Packard 8453 diode-array spectrophotometer. The absorbance data were analyzed globally using SPECFIT (Spectrum Software Associates). ^{1}H NMR spectra were collected on a Bruker 300 MHz spectrometer.

Dissociation Constant Measurements. Schiff base dissociation constants were measured by variation of amino acid or amine concentration in a series of solutions of constant volume and PLP concentration. Spectra (250−600 nm) were collected and analyzed globally by SPECFIT. The reactions were allowed to proceed to >5 half-lives of the slowest kinetic phase before spectra were recorded. The reactivity of MAM precluded equilibrium measurements, and the apparent dissociation constant reported in Table 1 was obtained from the hyperbolic dependence of the decarboxylation rate constant on MAM concentration.

Dissociation constants for Al^{3+} binding to the Schiff bases at pH 5 were measured using the pH jump described above. Equilibrium spectra (250−600 nm) were obtained after >5 half-lives of the slowest kinetic phase, and were analyzed by SPECFIT to determine the values of K_d. As with Schiff base formation, the reactivity of MAM required the use of kinetic data to obtain an apparent K_d for Al^{3+}. Rate constants for the first phase of the reaction of Al^{3+} with MAM−PLP in pH jump experiments (0.2 M MAM) showed a hyperbolic dependence on Al^{3+} concentration and were used to obtain the values reported in Table 1.

Rate Constant Measurements. Schiff base formation rate constants reported in Table 2 were measured in reactions containing 0.2 M potassium acetate, 0.2 M amino acid, 0.1 mM PLP, and 0.2 M potassium chloride at 25 °C. Spectra (250−500 nm) were collected versus time and analyzed globally using SPECFIT.

Metal ion binding rate constants were measured using a pH jump from 8 to 5. This allows saturation of PLP with amino acid at high pH followed by rapid metal ion binding and slow Schiff base hydrolysis at low pH. Reaction conditions were identical to those for Schiff base formation except various concentrations of Al^{3+} were included in the potassium acetate buffer. The Al^{3+} binding rate constants reported in Table 2 are maximal values obtained from hyperbolic fits of observed rate constants versus metal ion concentration.

MAM decarboxylation in the presence of Zn^{2+} or Ga^{3+} was measured using the pH jump method with 0.2 M MAM and 0.2 M potassium acetate, pH 5 and 4.5, respectively. The Ga^{3+} reactions could only be followed at pH 4.5 or lower due to precipitation of gallium.

Alanine and ethylamine transamination and AIB decarboxylation were conducted at pH 5 using 1 M amino acid or amine in the presence or absence of 15 mM Al^{3+}. No pH jump was used since full equilibration of Schiff base formation and metal ion binding occurs on the time scale of transamination and decarboxylation. The AIB and ethylamine reactions were extremely slow and were followed for only ∼10% of the reaction. The rate constants were obtained by fitting exponential curves in which the amplitude of the slow kinetic phase was fixed at the change in absorbance expected for complete PLP reaction.

Product Analysis. Product analysis was performed by quenching reaction aliquots with sufficient potassium hydroxide to neutralize reactants and buffer and give a final concentration of 0.5 M KOH. The base quench rapidly hydrolyzes any Schiff base present. Absorbance spectra were measured to determine the amount of PLP or PMP by comparison to the measured extinction coefficients of the pure compounds under similar conditions.

^{1}H NMR (D_2O, 0.5 M KOD) was used in some cases to validate the UV−vis product analysis. For PLP, ^{1}H NMR (0.5 M KOD, D_2O at 4.8 ppm): 10.4 (C4′-H), 7.64 (C6−H), and 2.34 (C2′-H_3). For PMP, ^{1}H NMR (0.5M KOD, D_2O at 4.8 ppm): 3.76 (C4′-H_2), 7.57 (C6−H), and 2.32 (C2′-H_3).

Results

Schiff Base and Metal Ion Dissociation Constants. Table 1 summarizes the dissociation constants for Schiff base formation between amino acids or amines and PLP, as well as for the binding of Al^{3+} to these Schiff bases. The stability of the Schiff bases decreases with decreasing pH as previously observed.[12] On the other hand, the apparent affinity of Al^{3+} for the AIB Schiff base increases with decreasing pH, in agreement

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Table 1. Dissociation Constants for Schiff Base Formation and Al^{3+} Binding[a]

reactants		K_d (mM)	
A	B	pH 5	pH 9
AIB	PLP	3000(100)	26(2)
MAM	PLP	650(100)[b]	
Alanine	PLP	1700(40)	1.2(0.03)
Ethylamine	PLP	310(10)	
Al^{3+}	AIB−PLP	0.023(0.007) 2(1)[b]	0.34(0.01)
Al^{3+}	MAM−PLP	2.3(2)[b]	0.44(0.3)[b]
Al^{3+}	Ala−PLP	0.034(0.009)	
Al^{3+}	EA−PLP	2.0(0.1)	

[a] Errors are given in parentheses. Reaction conditions: 25 °C in the dark, 0.1 mM PLP, 0.2 M KCl, variable concentration of reactant A, and either 0.2 M potassium acetate (pH 5) or 0.1 M CHES (pH 9). Titrations with Al^{3+} at pH 9 contained 1 M AIB, 1 M ethylamine, or 0.2 M MAM. Titrations with Al^{3+} at pH 5 were performed via pH jumps from pH 8 with 0.2 M amino acids or amine. [b] Kinetically determined values. The apparent K_d for MAM was determined from hyperbolic fits of observed decarboxylation rate constants versus concentration (Figure 3). Al^{3+} K_d values were obtained from hyperbolic fits of the rate constants for the first metal ion binding kinetic phase versus concentration. The Al^{3+}/MAM data at pH 5 were obtained in pH jump experiments in which Schiff base formation was maximized by preforming it at pH 8.

Table 2. Rate Constants for Schiff Base Formation and Al^{3+} Binding[a]

reactants		$k^{SB} \times 10^2$ (s^{-1})	$k_1^{met} \times 10^2$ (s^{-1})	$k_2^{met} \times 10^2$ (s^{-1})
A	B			
AIB	PLP	1.4 (0.1)		
MAM	PLP	2.2 (0.1)		
Al^{3+}	AIB−PLP		7.9 (0.6)	0.10 (0.01)
Al^{3+}	MAM−PLP		3.9 (0.8)	0.4 (0.1)

[a] Reaction conditions: 25 °C in the dark, 0.1 mM PLP, 0.2 M amino acid, 0.2 M KCl, 0.2 M potassium acetate, pH 5. Metal ion binding kinetics were measured using a pH jump method that involved preforming the Schiff base at pH 8, followed by addition of potassium acetate and various concentrations of Al^{3+} to give pH 5. The metalation rate constants are k_{max} values obtained from plots of k_{obs} versus Al^{3+} concentration. The k_{max} value for AIB Schiff base formation at pH 5 is 0.05 ± 0.01 s^{-1}.

with previous studies.[13] The kinetically determined apparent K_d values for Al^{3+} binding to the MAM Schiff base were obtained from the first kinetic process, and thus probably represent dissociation constants of initial encounter complexes that subsequently isomerize to the final forms. In agreement with this proposition, the kinetically determined apparent K_d at pH 5 for Al^{3+} binding to the AIB Schiff base is much larger than the equilibrium value and is, within error, identical to that for MAM. The K_d for Al^{3+} binding to the ethylamine Schiff base is ~100-fold larger than that for the AIB Schiff base, in accord with previous observations.[14] This is undoubtedly due to the absence of the carboxylate ligand to the metal ion in the Schiff base.

Schiff Base Formation and Metal Ion Binding Rate Constants. The data in Table 2 show that, at concentrations of AIB (0.2 M) high enough to saturate PLP at pH 8 (the pH at which Schiff base formation is allowed to occur before jumping the pH to 5 in reactions containing metal ion), metal ion binding at pH 5 is fast relative to Schiff base hydrolysis. On the other hand, the data for MAM show that Schiff base hydrolysis is only 2-fold slower than metal ion binding. The goal was to measure the rate constants for decarboxylation of PLP fully bound by amino acid and metal ion. Therefore, higher concentrations (1.8 M MAM, 1 M AIB) were used in the experiments in which metal ion dependent decarboxylation rate constants were measured (Table 3) to ensure nearly complete complexation of PLP as the metal ion bound MAM Schiff base at low pH.

The binding of metal ions (Al^{3+}, Zn^{2+}, or Ga^{3+}) to the Schiff bases exhibited multiple kinetic phases between pH 5 and pH 9, suggesting the fast formation of an encounter complex followed by slow isomerization. Figure 1 presents data obtained in the reaction of the AIB Schiff base with Al^{3+} at pH 5. The kinetic traces given in Figure 1A clearly show the two-exponential nature of the reaction. The spectra of the three species involved in the two-exponential process are shown in Figure 1B. The Schiff base (species A) has a typical absorbance maximum of ~420 nm, while the intermediate metalated Schiff base has a major peak at ~310 nm, and the final isomerized metalated Schiff base absorbs maximally at ~370 nm, in agreement with previous findings.[13]

The binding of Ga^{3+} (5 mM) to the AIB Schiff base exhibited three kinetic phases: $k_1^{met} = 6.0(0.4) \times 10^{-2}$ s^{-1}, $k_2^{met} = 4.5(0.1) \times 10^{-3}$ s^{-1}, $k_3^{met} = 1.5(0.1) \times 10^{-3}$ s^{-1}. Two Ga^{3+} binding phases were distinguished from MAM decarboxylation: $k_1^{met} = 3.0(0.1) \times 10^{-2}$ s^{-1}, $k_2^{met} = 1.8(0.1) \times 10^{-3}$ s^{-1}.

The first phase in Zn^{2+} binding to either the AIB or MAM Schiff base is complete in the mixing time (5−10 s). The rate constant for the slower phase for Zn^{2+} (5 mM) binding to the AIB Schiff base was $4.0(0.1) \times 10^{-3}$ s^{-1}, while that for binding to the MAM Schiff base was $2.3(0.1) \times 10^{-2}$ s^{-1}.

Decarboxylation and Transamination Rate Constants. Figure 2 presents data for the reaction of MAM with PLP in the presence of Al^{3+}. Three methods of analysis (continuous spectrophotometric monitoring, base quench followed by NMR, and base quench followed by spectrophotometric coenzyme determination) yield rate constants for the decrease of PLP or the increase of PMP that are in good agreement. Decarboxylation thus occurs as the slowest kinetic process in reactions containing metal ion and the most reactive amino acid. The data in Figure 3 show the effect of 15 mM (saturating) Al^{3+} on the rate constant for decarboxylation of the MAM Schiff base. The inclusion of the metal ion has essentially no effect on the rate of MAM decarboxylation.

Spectrophotometric data for the decarboxylation of AIB and the transamination of ethylamine are shown in Figure 4. Data obtained from base-quenched aliquots are in agreement with those obtained directly for alanine, AIB, and ethylamine. The inclusion of Al^{3+} in the reactions greatly slows AIB decarboxylation (125-fold) and slows ethylamine transamination to a lesser extent (4-fold). The transamination of alanine is slightly inhibited by Al^{3+} (1.3-fold). The rate of MAM decarboxylation decreased slightly with increasing concentrations of Ga^{3+}, Zn^{2+}, and several other metal ions examined.[1]

Table 3 collects the rate constants for decarboxylation and transamination in the presence and absence of Al^{3+}. The rate constants for the metal ion free reactions have been corrected for subsaturating amounts of amino acid or amine on the basis of the dissociation constants reported in Table 1. The reported values are thus for the Schiff bases. Previous reports demonstrated that dilute solutions of PLP analogues in strongly buffered solutions, as here, yield pyruvate quantitatively from

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Table 3. Rate Constants for Decarboxylation and Transamination in the Presence and Absence of Al^{3+} [a]

	$k \times 10^6$ (s^{-1})		$k \times 10^6$ (s^{-1})	
	pH 5		pH 5	pH 8
		Decarboxylation		
AIB−PLP[b]	3.9(0.3)	MAM−PLP[c]	10000(1000)	460(20)
AIB−PLP + Al^{3+}	0.031(0.003)	MAM−PLP + Al^{3+} [c]	9800(800)	450(10)
		Transamination		
alanine−PLP[b]	70(1)	ethylamine−PLP[b]	0.93(0.01)	
alanine−PLP + Al^{3+}	54(1)	ethylamine−PLP + Al^{3+}	0.25(0.04)	

[a] Reaction conditions: 25 °C in the dark, 0.1 mM PLP, 0.2 M KCl, 0.2 M potassium acetate (pH 5) or 0.2 M TEA (pH 8), and 15 mM Al^{3+} for the metal ion containing reactions. Errors are given in parentheses. [b] Rate constants for the reactions of AIB, alanine, and ethylamine (each present at 1 M) without Al^{3+} have been adjusted to 100% PLP saturation on the basis of the dissociation constants given in Table 1. [c] k_{max} values from hyperbolic fits of observed rate constants versus either MAM or Al^{3+} concentration. In the presence of Al^{3+}, MAM was present at 1.8 M at pH 5 and 0.2 M at pH 8. Data at pH 5 were measured using a pH jump from 8 to 5 to maximize Schiff base formation.

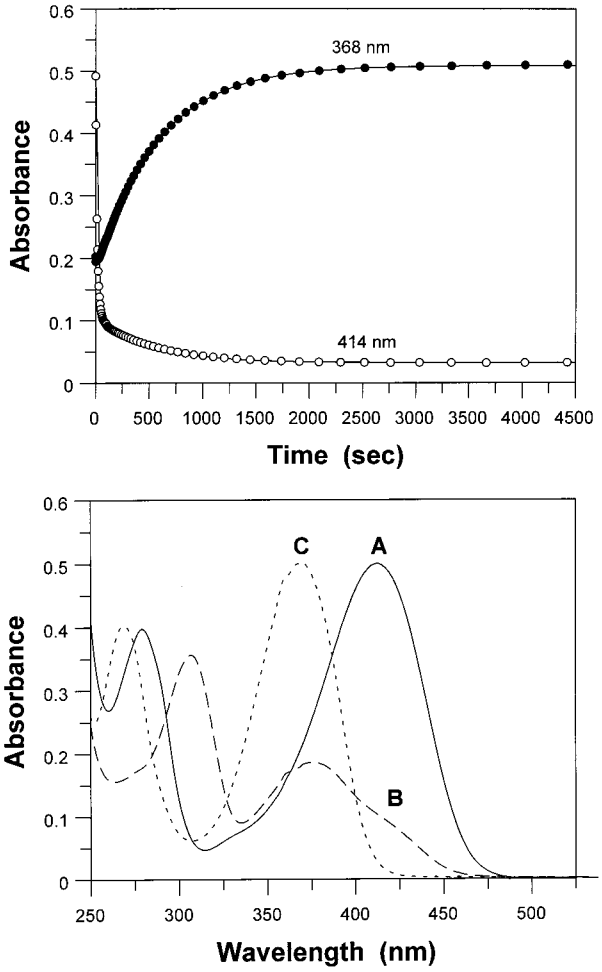

Figure 1. (A, top) Time courses for the reaction of the preformed AIB−PLP Schiff base with 15 mM Al^{3+} at pH 5. Biphasic kinetics are clear from the data. (B, bottom) Spectra of the species observed in the Al^{3+} binding reaction: A, Schiff base (λ_{max} = 414 nm), B, intermediate metalated Schiff base (λ_{max} = 374 nm); C, final Al^{3+}−Schiff base complex (λ_{max} = 368 nm).

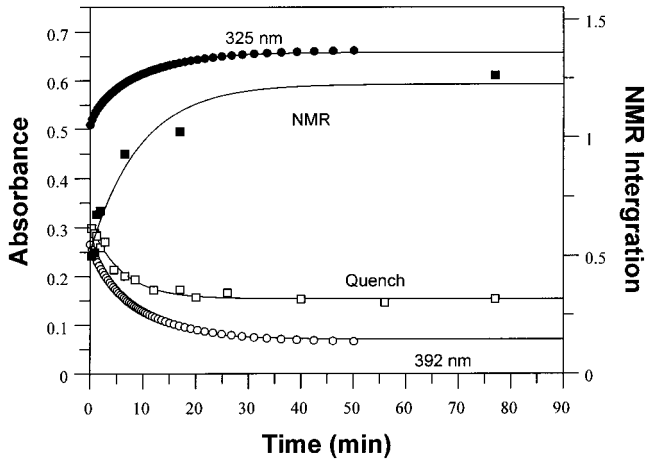

Figure 2. Time course for absorbance changes at 325 nm (PMP formation) and 392 nm (PLP loss) and of PMP formation detected by NMR and PLP loss detected by base quenching with spectrophotometric quantification for MAM decarboxylation in the presence of 4 mM Al^{3+} at pH 5.

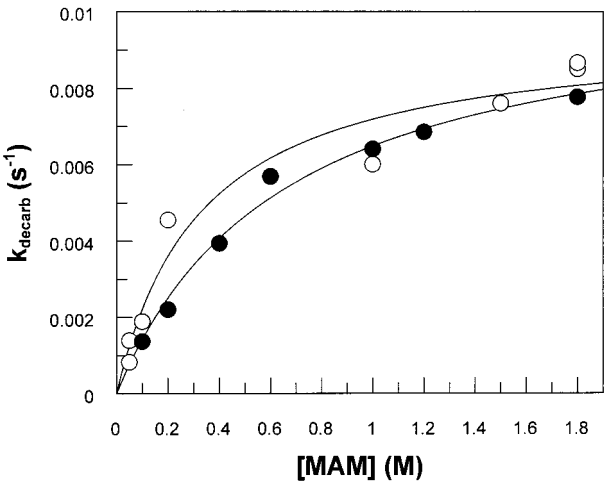

Figure 3. Observed rate constants for MAM decarboxylation at pH 5 in the presence (solid circles) and absence (open circles) of 19 mM Al^{3+}.

alanine via transamination[9] and pyruvate and alanine from the decarboxylation of MAM.[15]

The rate constants reported in Table 3 for both MAM decarboxylation and alanine transamination catalyzed by PLP are in excellent agreement with those previously measured under similar conditions. Thanassi[15] reported a value of $\sim 4 \times 10^{-3}$ s^{-1} for the 5-deoxypyridoxal catalyzed decarboxylation of MAM. Auld and Bruice[9] reported rate constants for 3-hydroxypyridine-4-aldehyde catalyzed transamination of alanine from which one can calculate an expected value of 7×10^{-5} s^{-1} in half-neutralized 0.2 M acetate buffer. The transamination of γ-aminobutyrate, mechanistically similar to that with ethylamine, was previously reported to be much slower than that of alanine,[16] consistent with the 75-fold difference in rate constants reported in Table 3.

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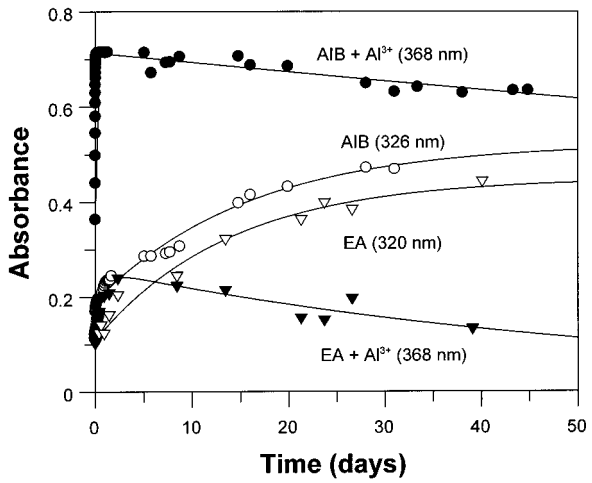

Figure 4. Time courses for the decarboxylation of AIB and transamination of ethylamine in the absence and presence of 16 mM Al^{3+}.

Discussion

Quinonoid Intermediate Stability. Several reports have described the observation of dihydropyridine tautomers of Schiff bases (quinonoid intermediates) between amino acids and PLP analogues by their characteristic long-wavelength (~500 nm) absorbance. Schirch and Slotter[17] and Abbott and Bobrik[18] reported observing in water a quinonoid intermediate from diethylaminomalonate and pyridoxal, while Maley and Bruice[19] found a similar species in water during the reaction of 1-methyl-4-formylpyridine with glycine, alanine, and glycine ethyl ester. This species has been observed in reactions of the ethyl esters of alanine in methanol in the presence of Al^{3+} by Matsumoto and Matsushima.[20]

Abbott and Martell[21] examined the reaction of the 1:1 Al^{3+}-bound alanine Schiff bases by NMR. These authors proposed that a stable quinonoid intermediate is formed in the course of the reaction due to the strong electrostatic effect of the trivalent metal ion as compared to the proton. The possibility that the decarboxylation of MAM in the presence of Al^{3+}, which is much faster than the transamination of alanine, produces a stable quinonoid intermediate was therefore considered.

Spectra collected in the course of the PLP−MAM:Al^{3+} reaction have no detectable absorbance band at wavelengths longer than 450 nm (indicative of a quinonoid intermediate), as was observed in the references cited above. Formation of a stable quinonoid intermediate after rapid CO_2 loss equates to protonation of the quinonoid intermediate being the rate-determining step in the conversion of the PLP−MAM:Al^{3+} chelate to either the pyruvate ketimine or the alanine aldimine (which corresponds to protonation on C4′ of the coenzyme or Cα of the amino acid, respectively). If a stable quinonoid that does not absorb above 450 nm is very rapidly formed and protonation of it is rate-determining, then PLP loss and PMP formation as determined by the quench methods would correspond to one of the early processes observed in the spectral data obtained directly from the unquenched reaction. This is not the case; rate constants from quench data coincide with those obtained spectroscopically from the slowest kinetic process. Thus, loss of CO_2 from the final PLP−MAM:Al^{3+} complex is the rate-determining step in MAM decarboxylation. By extension, CO_2 loss or deprotonation of the PLP−amino acid:Al^{3+} complexes of AIB, alanine, and ethylamine are assumed to be rate-determining since these substrates are much less reactive. In support of this assumption, no absorption bands above 450 nm were observed in any of these reactions.

Al^{3+} Inhibits Decarboxylation and Transamination of Metalated Schiff Bases. Metal ion catalysis of transamination[7,8] has been reported, while Kalyankar and Snell[22] reported a ~2-fold reduction in AIB decarboxylation by metal ions. Metal ions were reported to inhibit slightly β-elimination reactions.[23] The rate constants for the reactions of metal ion free Schiff bases and their 1:1 Al^{3+} chelates reported in Table 3 give a direct measure of the effect of Al^{3+} solely on the central chemical step (i.e., CO_2 loss or deprotonation), unlike the previous studies cited above.

The data indicate that protonated Schiff bases are generally more reactive than Al^{3+}-bound Schiff bases. Ethylamine reacts 4-fold faster and alanine reacts 1.3-fold faster in the absence of Al^{3+} than in its presence. Decarboxylation of the AIB Schiff base shows a larger (125-fold) effect due to the chelation of the reactive carboxylate group by the metal ion. Carboxylate chelation would promote deprotonation (i.e., transamination) stereoelectronically by orienting the Cα−H bond more nearly perpendicular to the pyridine ring plane, but inhibit CO_2 loss both stereoelectronically and electrostatically. The MAM Schiff base, with two carboxylate groups, would incur a combination of favorable stereoelectronic effects and unfavorable electrostatic effects for CO_2 loss, which is apparent in the lower rate constant ratio of 1 in the absence and presence of Al^{3+}, as compared to 125 for AIB.

The previously observed rate enhancements due to the presence of metal ions can now safely be ascribed simply to the enhancement of Schiff base formation by the metal ions in solutions containing subsaturating concentrations of amino acids. The inhibitory effect of metal ions on decarboxylation and transamination of the Schiff bases examined here provides a viable explanation for the lack of observation of PLP dependent enzymes that make direct mechanistic use of metal ions in the central chemical steps.

Pyridine Nitrogen Protonation. The pK_a of the pyridine nitrogen of Schiff bases formed between PLP and α-amino acids is in the range 5.9−6.7,[24] while that for metalated Schiff bases is ~7.4.[12] The MAM data in Table 3 at pH values of 5 and 8 thus provide a rough comparison of the reactivities of the pyridine nitrogen protonated and unprotonated Schiff bases. For both the metal ion free and Al^{3+}-bound forms, the protonation of the pyridine nitrogen has only a ~20-fold effect, which is small compared to the total rate acceleration due to PLP in nonenzymatic reactions (see below).

A similar 20-fold effect was observed by Maley and Bruice[25] in the comparative transamination of alanine by 3-hydroxypyridine-4-aldehyde vs 1-methyl-3-hydroxypyridine-4-aldehyde, where quaternization in the latter enforces a positive charge on the nitrogen. In their studies of the transamination of alanine by 3-hydroxypyridine-4-aldehyde, Auld and Bruice[9] made the

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interesting observation that protonation of the α-carboxyl group has an effect equal to protonation of the pyridine nitrogen, amounting to a ∼75-fold rate enhancement. Thus, the strict conservation in a very large number of PLP dependent enzymes of an arginine residue that interacts intimately with the substrate α-carboxyl group[26] may reflect not simply binding interactions, but also mechanistic requirements. A quantitatively coincidental factor of 75-fold is observed here for the increase in Schiff base reactivity toward transamination due to the presence of an unprotonated α-carboxylate function (alanine vs ethylamine; Table 3).

The relatively small effect of pyridine nitrogen protonation in the nonenzymatic reactions suggests that, as has been found in computational studies,[27,28] the protonated azaallylic function (Schiff base) may be the dominant factor in stabilization of carbanions generated on the substrate carbon bearing the nitrogen atom of the Schiff base. In this regard, it is significant that a very wide variety of carbonyl compounds,[29] including formaldehyde,[30] can function as catalysts of amino acid decarboxylation. The existence of the pyruvoyl-dependent decarboxylases also highlights the ability of a Schiff base derived from a simple carbonyl function to stabilize well an azaallylic carbanion.

Comparison of Enzymatic and Nonenzymatic Rates. The decarboxylation of AIB by the PLP dependent enzyme dialkylglycine decarboxylase occurs with a maximal rate constant of 25 s^{-1} at pH 8 and 25 °C.[31] This value is $\sim 10^8$-fold greater than the PLP dependent nonenzymatic reaction would be, assuming a similar 20-fold lower decarboxylation rate constant at pH 8 as found with MAM. The same enzyme catalyzes the transamination of alanine with a maximal rate constant of 22 s^{-1},[31] giving a $\sim 10^7$-fold increase over the PLP dependent nonenzymatic rate. The transamination of γ-aminobutyrate by the *E. coli* isozyme of γ-aminobutyrate aminotransferase occurs with a k_{cat} value of ∼50 s^{-1} (J. Langston and M. D. Toney, unpublished results), giving a $\sim 10^9$-fold increase over the PLP dependent nonenzymatic rate.

Radzicka and Wolfenden[32] measured nonenzymatic rate constants for a variety of reactions that are catalyzed by enzymes and showed that OMP decarboxylase provides an enormous rate enhancement of $\sim 10^{17}$-fold. The rate constant for either alanine or glycine spontaneous decarboxylation at neutral pH and 25 °C, obtained by extrapolation from high temperatures, is $\sim 2 \times 10^{-17}$ s^{-1} and rather insensitive to pH in the neutral range (Snider and Wolfenden, *J. Am. Chem. Soc.*, in press). One can calculate from this and the data in Table 3 that, in the absence of enzyme, PLP catalyzes the decarboxylation of AIB by $\sim 10^{11}$-fold at pH 5 and by $\sim 10^{10}$-fold at pH 8. This impressive rate acceleration by PLP alone is equal to or better than that of many enzymes.[32]

Thanassi[33] reported a rate constant of $\sim 10^{-7}$ s^{-1} for the spontaneous decarboxylation of aminomalonate at pH 5. Compared to the value of 10^{-2} s^{-1} for the PLP catalyzed decarboxylation of 2-methylaminomalonate at the same pH, this represents only a $\sim 10^5$-fold rate acceleration by PLP for this intrinsically much more decarboxylation reactive amino acid, in accord with expectation.

The total decarboxylation rate enhancement at pH 8 due to the enzyme−PLP complex (i.e., holoenzyme) is $\sim 10^{18}$-fold. Thus, PLP dependent decarboxylases and OMP decarboxylase are currently the best known biological rate enhancers, as determined from first-order rate constant ratios. The $\sim 10^8$-fold component of this due to the protein portion of the holoenzyme is in the middle of the known range of coenzyme independent enzymes;[32] in other words, the protein portion does a rather average job at enhancing the decarboxylation rate constant. The enormous rate acceleration of the holoenzyme is only possible due to the high efficiency of the PLP coenzyme itself, assuming its efficacy in the enzyme active site is at least equal to that in solution.

An additional and possibly equally important role of the protein in PLP dependent enzymes is to direct the reaction of the external aldimine intermediate (i.e., the enzyme-bound PLP−substrate Schiff base) along a specific reaction pathway, since this intermediate has multiple reaction pathways available to it. Dunathan's[34] elegant proposal of stereoelectronic control of reaction specificity combined with specific placement of auxiliary catalytic functional groups, mechanisms not available to the nonenzymatic reactions, likely accounts for the observed high specificities of PLP dependent enzymes.

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