The Role of the Delocalized α,α'-Carbanion in Vitamin B_6 and Al(III) Catalyzed Transamination of α-Amino and α-Keto Acids

PATRICK A. TAYLOR and ARTHUR E. MARTELL*

Department of Chemistry, Texas A & M University, College Station, Tex. 77843, U.S.A.

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Abstract

The rates of the transamination reactions of α-amino acids and α-keto acids were followed by measurement of the 200 MHz proton NMR spectra of solution species as a function of time. Reaction systems measured in D_2O at 10 °C consisted of 1:1:1 molar ratios of pyridoxal:α-amino acid:Al(III) or pyridoxamine:α-keto acid:Al(III). Amino and keto acids employed are alanine, α-aminoisobutyric acid, valine, phenylglycine, pyruvic acid, and α-ketobutyric acid. A negatively charged deprotonated Schiff base coordinated to Al(III) was detected in all systems that undergo transamination (*i.e.*, except α-aminoisobutyric acid). The intermediate resembles the aldimine Al(III) chelate with NMR resonances shifted upfield in accordance with its greater negative charge. Its equilibrium concentration is reached in the time required to reach transamination equilibrium and is maintained in solution at a *ca.* 10–20% of the aldimine Schiff base concentration.

Introduction

The mechanism of enzymatic and nonenzymatic vitamin B_6 catalyzed transamination of α-amino and α-keto acids proposed by Metzler *et al.* [1] involves the formation and interconversion of aldimine and ketimine Schiff bases. Spectrophotometric characterization of such transamination intermediates in methanol has been reported by Matsushima and Martell [2, 3] and for aqueous solution by Bruice *et al.* [4–13]. Nuclear magnetic resonance characterization in aqueous solution has been reported by Gansow and Holm [14–17] and by Abbott and Martell [18–21].

In the case of the pyridoxal-catalyzed transamination reactions of amino acids considerable interest has been shown in the identification and characterization of an intermediate resulting from the dissociation of the α-amino acid proton of the Schiff base in the absence of and in the presence of metal ions. Characterization of intermediates obtained by dissociation of the α-proton from the amino acid in the aldimine has been described for model systems by Maley and Bruice [13]. In their study it was observed that the dissociation of the α-proton from the Schiff base formed from glycine or alanine with 1-methyl-4-formyl pyridinium iodide produces a species absorbing in the visible region of the spectrum (500–600 nm) suggesting the formation of a dihydropyridine type tautomer of the aldimine Schiff base. The dihydropyridine (DHP) intermediate was suggested by Metzler *et al.* [1], and later by Schirch and Jenkins [22] as the absorbing species with a maximum near 505 nm in enzyme-substrate complexes of vitamin B_6 enzymes. A number of other investigators have also reported enzyme-substrate complexes of vitamin B_6 absorbing near 500 nm [23–25].

The NMR spectra of pyridoxal (PL) and pyridoxamine (PM) have been studied as a function of pD and the principal species present have been identified by Gansow and Holm [14][§]. Transamination reactions of alanine [16] and pyruvate [15] have also been studied at various pDs without metal ion and with coordinated Zn(II) and Al(III). The major species were also identified in these systems.

Abbott and Martell established that free Schiff base is not observed as a separate entity below pD 7 in the absence of metal ions [26]. Therefore, resonances observed with chemical shifts between 8.92 and 9.01 ppm must be something other than free Schiff base. Various possible structures to account for this unassigned resonance, as well as three additional unassigned resonances farther upfield were explored [27] and the following six observations on the PL–alanine–Al(III) system were made. (1) The appearance of the unassigned chemical shifts roughly parallels the appearance of PM. (2) These chemical shifts decrease in intensity as transamination from aldimine to ketimine nears completion. (3) The structure must be similar to that of the aldimine because of similar chemical shift environments. (4) The 4′-CH

*Author to whom correspondence should be addressed.

§ 1.24 ppm was added to the chemical shifts reported by Gansow and Holm because of the use of DSS as a reference standard in place of t-butyl alcohol.

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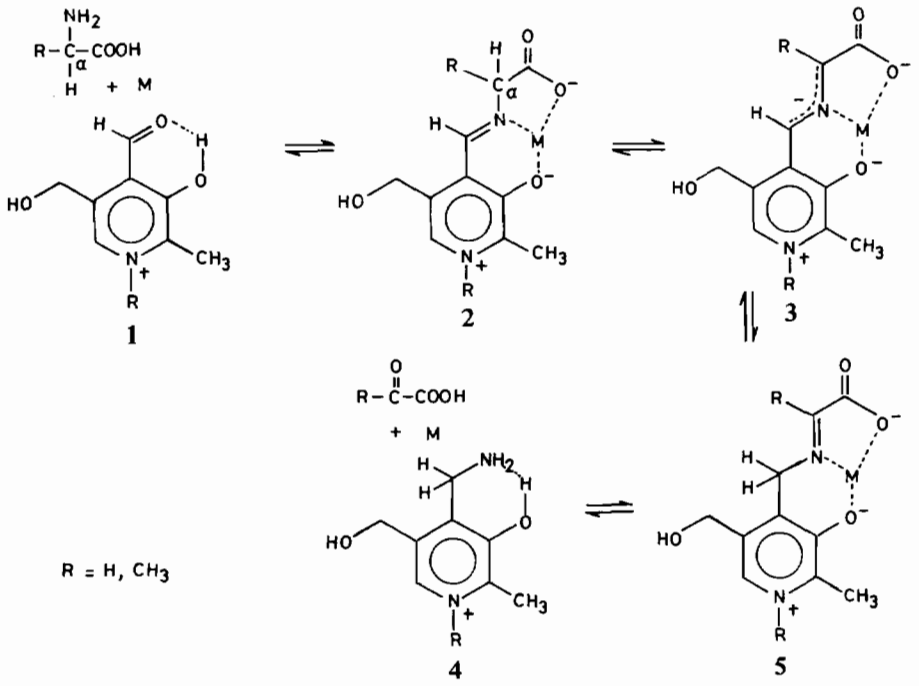


Scheme 1. Proposed mechanism for pyridoxal and metal ion-catalyzed transamination of α-amino acids.

TABLE I. Comparison of the Chemical Shifts with Respect to DSS of 1:1 Schiff Base–Aluminum(III) Complexes

1:1[a] Aldimine complexes	Intermediate[a] (αDI)	1:1[b] Ketimine complex
3.05 (2'-CH_3)	3.11	1.52 (2'-CH_3)
5.36 (5'-CH_2)	5.20	[c]
8.31 (6-CH)	8.35	7.63 (6-H)
9.31 (4'-CH)	8.99	4.86 (4'-CH_2)

[a]Ref. 28. [b]Ref. 17. The ketimine resonances were reported with respect to tertiarybutyl alcohol and have been adjusted to be comparable with the other resonances in this Table by adding 124 Hz. [c]Obscured by the resonance of the isotopic impurity (HOD) of the solvent.

chemical shift of the complex is found farther upfield than any previously reported aldimine, yet not high enough to be associated with the saturated 4'-CH_2 of the ketimine. (5) Polynuclear complexes and mixed ligand complexes were excluded as possible structures. (6) The intermediate is formed in appreciable concentration and is not an unstable intermediate representing an activated state of a metal complex. From these observations they postulated that these NMR resonances may be due to the formation of an intermediate resulting from dissociation of the α-proton of the pyridoxal–alanine Schiff base, and proposed that this previously unobserved intermediate is characterized by a delocalized negative charge at the azomethine group stabilized at that position by the coordinated metal ion.

The reaction sequence for pyridoxal and pyridoxamine-catalyzed transamination in model systems, illustrated in Scheme 1, has been proposed recently [30, 31]. The α-deprotonated intermediate (αDI) species 3 was suggested as the common intermediate for both forward and reverse transamination. With $M^{n+} = Al^{3+}$, this intermediate would be the same as the one suggested by Abbott and Martell [27] on the basis of the NMR evidence presented in Table I.

In the present study the variation of the chemical shifts of this new intermediate with time was measured to provide information on its constitution and its probable involvement in the mechanism of vitamin B_6-catalyzed transamination. A brief communication comparing the observed forward and reverse transamination rates with the rates of formation of the suggested deprotonated intermediate for the PL–alanine Schiff base complexes has been published [28]. The present paper extends and supplements this previous report by providing experimental details

TABLE II. Chemical Shifts of Major Species Found in Systems Studied[a,b]

Species	pD	6-H Proton	2'-CH_3 Protons	4'-CH or 4'-CH_2 Protons	5'-CH_2 Protons	α-H Proton
Pyridoxal	5.0	7.91	2.58	6.66	5.24[c]	
Pyridoxamine	5.1	7.50	2.33	4.16	4.58	
Aldimine (1:1 complex)	5.0	7.90	2.64	9.00	[d]	4.18
Ketimine (1:1 complex)	5.1	7.80	2.21	4.38	4.67	
αDI (1:1 complex)	5.0	7.86	2.75	8.76	5.60	
Aldimine (2:1 complex)	5.0		A 2.53	9.19 (broad)[e]		
			B 2.44			
			C 2.27			
			D 2.03			
αDI (2:1 complex)	5.0			8.65 (broad)[e]		

[a]Chemical shifts relative to 3-(trimethylsilyl)-1-propanesulfonic acid (DSS). [b]Chemical shifts vary with p[D] as shown in this investigation and by other investigators [16, 17]. [c]AB system centered at this chemical shift. [d]Obscured by the HOD isotopic impurity of the solvent. [e]The broadness of these 2:1 4'-CH peaks is due to the several stereoisomers that exist for the bis-complexes [20].

and by extending the study to several additional amino and keto acids. This study includes both aldimine and ketimine formation, the influence of pD, and the effect of the nature of the amino acid on rates of formation of the intermediate and the reaction product.

Experimental

NMR spectra of D_2O solutions of the systems under investigation were measured with a Varian XL-200 spectrometer. The probe temperature was 10.0 ± 0.5 °C unless otherwise stated. Chemical shifts are reported with respect to 3-(trimethylsilyl)-1-propanesulfonic acid (DSS). Typical chemical shifts employed to follow the concentrations of major species in D_2O solution are given in Table II. The p[D] values of the experimental solutions followed by NMR were determined with a Beckman Model 1019 pH meter equipped with a Sargent Welch glass–silver–silver chloride combination electrode. The relationship p[D] = p[H] + 0.40 was used to calculate p[D] [29]. For the purposes of this publication p[D] and p[H] represent $-\log[D^+]$ and $-\log[H^+]$, respectively.

The 0.100 M experimental solutions were prepared with a 1:1:1 molar ratio of pyridoxal:amino acid:metal ion. Aluminum sulfate hydrate was placed in D_2O and evaporated to near dryness three times before use. Amino acid, pyridoxal and aluminum sulfate were mixed at 0 °C in D_2O and the p[D] was adjusted with NaOD. The solution was then placed into the probe where it was warmed to 10.0 ± 0.5 °C. Time zero is defined as the time at which the sample was placed in the probe . The time required (at 0 °C) to place the experimental mixture in the probe was approximately 5 min. For samples of p[D] 4–6 a p[D] increase of 0.2 p[D] units was observed while at p[D] 9 a p[D] decrease of 2.0 p[D] units was observed in most experimental runs.

Phenylglycine is soluble in D_2O only at very high or very low p[D]. Therefore, solutions containing phenylglycine were mixed at high p[D] without metal ion. Once all crystals were dissolved the p[D] was lowered to the desired p[D], the metal ion was added, and the p[D] was corrected once again. Any precipitate (formed in trace amounts) was filtered off with glass wool before NMR measurements were made.

The amino acids, keto acids, pyridoxal hydrochloride, and pyridoxamine dihydrochloride were of the highest commercial grade available. The D_2O was 99.8 per cent pure and the NaOD used was a 40% solution in 99+% D_2O from Aldrich. The DCl employed was a 20% solution in 99+% D_2O from Alpha Products.

Results and Discussion

The Effect of p[D] on Transamination in the 1:1:1 PL–Alanine–Al(III) System

The rates of transamination and αDI formation were followed by the integration of NMR resonances of the 4'-CH or 4'-CH_2 (see Table I) as a function of time at five pD values in the range from moderately acidic to moderately alkaline: 4.0, 5.0, 6.3, 9.5, and 10.5. Sample data and results are shown in Table III for three of these pD values.

The obvious effect of increasing p[D] was the increase in the rate of formation of aldimine, and a corresponding increase in the concentration of aldimine ultimately reached, reflecting the greater tendency for Schiff base formation as the p[H] (or p[D]) is

TABLE III. Percent of Species[a] Found in Various Systems Consisting of 1:1:1 Molar Ratios of Pyridoxal:Amino Acid:Al(III) or Pyridoxamine:Keto Acid:Al(III)

Amino or Keto acid, conditions	Elapsed time	Aldimine		Ketimine	αDI		Pyridoxal lost	Transamination products	Comments
		1:1	2:1		1:1	2:1			
Alanine	2 min	4	4		obs		8		
p[D] 5.0; 10.0 °C	1 h 2 min	7	2		obs		20		
	3 h 44 min	11	2	obs	2		39	obs	
	5 h 14 min	14	2	obs	5		46	obs	
Alanine	4 min	2					15		
p[D] 6.3; 10.0 °C	1 h 4 min	13		obs			28	obs	
	6 h 6 min	10		obs	4	2	48	obs	
Alanine	10 min	47	5		obs		4′-CH proton not		racemization predominates
p[D] 10.5; 10.0 °C	5 h 44 min	39			obs		observed because of deuteration		over transamination
Valine	35 min	33			2	2	33		
p[D] 5.4; 25.0 °C	2 h 35 min	47			5	10	57		
	5 h 22 min	42			6	10	71		
Phenylglycine	5 min	too low for NMR observation			too low for NMR observation		31		hemiacetaldimine 25
p[D] 5.20; 10.0 °C	5 h 56 min	obs		obs	obs		66	obs	hemiacetaldimine 31
2-Aminoisobutyric acid	20 min	73	7		no αDI		86	no transamination	
p[D] 5.0; 10.0 °C	2 h 20 min	48	10		no αDI		84	no transamination	
Pyruvate	15 min	2		25	2		NA	obs	
p[D] 5.0; 0 °C	2 h	8		11	12	obs	NA	obs	
	24 h 36 min	12	6	11	2	2	NA	obs	

[a]Percentages calculated by $[X]_t/[PAC]_i \times 100$ where $[X]_t$ is the integral height of the 4′-CH proton of the reported species, and $[PAC]_i$ is the initial integral height of PL + initial integral height of 4′-CH proton of aldimine.

increased, because of the high proton affinities of free amino acid and pyridoxal. Thus at pH 4.0 the concentration of aldimine was never higher than 3%, even over a long time period. While the loss of pyridoxal indicated that some transamination was taking place, the formation of pyruvate was not detected by NMR. This was due in part to the difficulty in integration resulting from the proximity of the CH_3 chemical shift of pyruvate to that of the 2′-CH_3 of PL and the β-CH_3 of alanine. Because of the low concentration of the aldimine, it was not surprising, in view of the observations described below, that the αDI was not detected under these conditions.

Although the aldimine chelate tends to dissociate in acid solutions, the diprotonated form of the Schiff base ligand strongly favors transamination over other pyridoxal-catalyzed reactions [30, 31]. Thus a balance between these two opposing tendencies is needed to best catalyze the transamination reaction. This balance seems to be partially achieved at p[D] 5.0. The aldimine was observed in two minutes and eventually reached approximately 15% of the components while the αDI became minimally detectable at about 2%, finally growing to the 5% level. The transamination product, pyruvate, was also observed but not integratable. It was identified by the methyl resonance of the hydrate (at 1.20 ppm) and the free acid (at 2.51 ppm). Free pyridoxamine was identified by resonances at 4.60 ppm (5′-CH_2) and 7.90 ppm (6-H).

The most favorable p[D] for transamination was found to be 6.3. In this case formation of aldimine and the αDI which followed occurred more rapidly than at p[D] 5.0, but the tendencies for the integrated NMR signals to drop off at longer time periods, which occurred at both p[D]s, was more pronounced at p[D] 6.3. This kind of decrease was also reported earlier [27] and is probably due to deuteration of the 4′-CH proton by recycling through the forward and reverse transamination reactions.

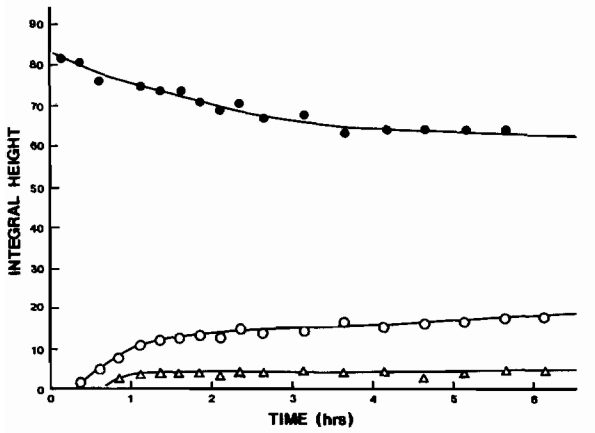


Fig. 1. Integral heights of 4′-CH proton resonance *vs.* time of a D_2O solution containing a 0.10 M pyridoxal, 0.10 M alanine, and 0.10 M Al(III) at p[D] 6.3. Where ● = 4′-CH of aldimine complex; ○ = 4′-CH of 1:1 αDI complex and △ = 4′-CH of 2:1 αDI complex.

The sequence of increase and decrease of concentrations of the aldimine and αDI complexes observed in this system (Fig. 1) is significant. The maximum concentration of aldimine complex occurred early and decreased with time, finally leveling off as equilibrium was reached. On the other hand the αDI complex was observed to increase in concentration to its maximum value as equilibrium was approached. Thus the αDI seems to be an intermediate in the transamination reaction, being formed at the expense of the aldimine complex, and initially in higher concentration than the ketimine complex. The integral heights measured for the formation of the αDI complex were plotted as described above. The first hour and fifteen minutes gave a straight line, indicating first order kinetics for initial formation of the αDI complex; $k_{obs} = 1.11 \times 10^{-4}\ s^{-1}$.

The 2:1 complex of the aldimine was observed; however, because its characteristic peaks are close to large peaks of other species, no quantitative data were obtained. Characteristic peaks for the 2:1 aldimine chelate at p[D] 6.3 are: 4′-CH 8.2 ppm; 2′-CH_3, A 2.29 ppm, B 1.96 ppm, C 1.88 ppm, D 1.63 ppm. In agreement with the work of Abbott and Martell [20], 2:1 Schiff base:Al(III) chelate formation is considerably enhanced in this p[D] range.

The reactions observed at p[D] 9.5 and 10.5 were relatively rapid, with formation of aldimine to its maximum concentration in 5–10 min. However, both transamination and racemization are slow at p[D] 9.5, and the concentrations of αDI and ketimine intermediate observed were very low. The slowness of both reactions was further indicated by the fact that the β-methyl doublets remained at appreciable concentrations through the reaction period.

At p[D] 10.5, however, the 4′-CH resonance disappeared quickly even when free pyridoxal was still present. Apparently, the loss of the 4′-CH resonance was due to deuterium exchange, in accordance with the fact that at this p[D] racemization is rapid. The fact that it is much more rapid than transamination was further indicated by the fact that pyruvate formation was not detected throughout the reaction period. The appearance of αDI (Table III) does not discriminate between these two reactions (racemization and transamination) since both of these reactions require α-deprotonation to produce an Al(III)-stabilized delocalized carbanion. In time the 6-position became deuterated, so that the methyl doublets collapsed to singlets. Also, as deuteration proceeded the αDI resonances broadened, preventing quantitative estimation by integration.

1:1:1 PM–Pyruvate:Al(III)

At p[D] 5.0, 0 °C, ketimine formation had reached its maximum concentration before the first measure-

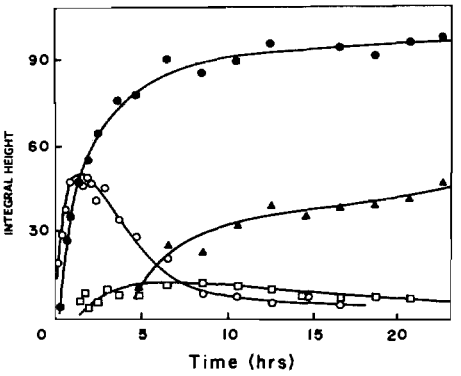


Fig. 2. Integral heights of 4'-CH proton of a 0.1 M D_2O solution containing a 1:1:1 molar ratio of pyridoxamine, pyruvate, Al(III) at p[D] 5.0 *vs.* time. Where ● = 1:1 aldimine complex 4'-CH; ▲ = 2:1 aldimine complex 4'-CH; ○ = αDI (1:1), □ = αDI (2:1).

ment was taken. As transamination occurred the percentage of ketimine dropped, and αDI and aldimine chemical shifts became detectable (Table I). Since the aldimine Schiff base complex is more stable than that of the ketimine, the transamination equilibrium is shifted toward aldimine. Transamination products were detected after 1 h 15 min. At that time αDI concentration exceeded that of the aldimine, but subsequently the aldimine concentration became greater than that of the αDI (Fig. 2), as equilibrium was approached. The initially higher concentration of the αDI and the gradual adjustment to the equilibrium concentration which is considerably lower than the aldimine complex is the consequence of its role as the intermediate in the transamination process. The observed subsequent, further slow decrease in the concentration of αDI is apparently due to deuteration.

Reactions with 2-Aminoisobutyric Acid

When parallel experiments were carried out with α-aminoisobutyric acid (Table III), it was found that for a 1:1:1 pyridoxal–amino acid–Al(III) system at p[D] 5.0, extensive aldimine formation occurred. Both the 2:1 and 1:1 Schiff base Al(III) chelates were formed, but no transamination products were detected. Also there was no indication of an αDI intermediate, in accordance with the requirement of a dissociable α-proton for αDI formation.

PL–Valine–Al(III)

In a 0.10 M D_2O solution containing 1:1:1 molar ratio of pyridoxal, valine, Al(III) at p[D] 5.0, 10.0 °C, aldimine formation was found to be slow; therefore, transamination and αDI formation are also slow. After 4 h 4 min some αDI formation was detected.

At room temperature (25.0 °C) and p[D] 5.4, thirty five minutes after mixing the resonances of valine and pyridoxal were observed, along with those of the aldimine Schiff base complex and some αDI

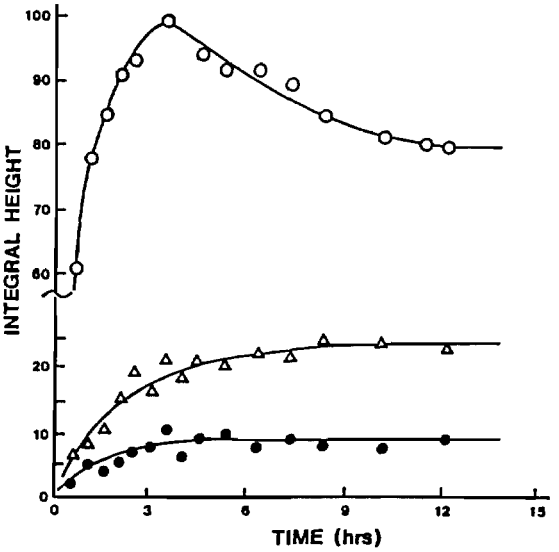


Fig. 3. Integral heights of 4'-CH resonance *vs.* time for D_2O solutions of 0.10 M pyridoxal, 0.10 M valine, 0.10 M Al(III), p[D] 5.0; where ○ = aldimine; ● = αDI (1:1); △ = αDI (2:1).

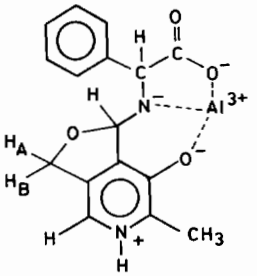
formation. As time proceeded the concentrations of aldimine and of the αDI and keto acid resulting from transamination increased. At this p[D] the system is very close to the p[D] where 2:1 complex formation had been reported previously (p[D] 5.5) [20]. Formation of the 2:1 complexes was confirmed by the increase in intensities of the four characteristic 2-CH_3 resonances of the 2:1 aldimine complex. Also, the αDI was observed in the 2:1 form, which is apparently preferred at this p[D]. Over the period of 12 h 9 min both 1:1 and 2:1 forms of the αDI steadily increased, while the concentration of aldimine reaches its maximum concentration at 3 h 30 min and then began to decrease. At about 12 h the system appeared to have reached transamination equilibrium and the further slow decreases in concentration were ascribed to deuteration of the 4'-CH proton as the reaction system cycled between the aldimine and ketimine Schiff bases.

From the above observations it was possible to follow the variation in species concentrations (Fig. 3) as the system approached equilibrium. First the aldimine Schiff base was formed in large amounts, pushing the equilibrium toward the less stable αDI, and subsequently toward the ketimine transamination products. As the system reached equilibrium each species was observed to reach a steady concentration. At the p[D] employed relatively high concentrations of the 2:1 aldimine complex were observed.

PL–Phenylglycine–Al(III) System

Five minutes after mixing of a 0.10 M solution containing a 1:1:1 molar ratio of pyridoxal, phenylglycine, and Al(III) at p[D] 5.20 and 10.0 °C, the

chemical shifts for free pyridoxal and phenylglycine indicated that little reaction had taken place. Also present was a singlet at 8.57 ppm which indicated that some aldimine formation had taken place. However, a doublet of doublets centered at 4.63 ppm and 4.68 ppm corresponding to non-equivalent 5′-CH_2 protons, suggest that the hemiacetaldimine (**6**) had also formed.



6. Proposed hemiacetaldimine of PL and phenylglycine

The observed coupling constant for the doublet of doublets, $J_{AB} = 6.1$ Hz, is lower than those of hemiacetals (pyridoxal $J_{AB} = 14$ Hz) [14] and hemiacetaldimines ($J_{AB} = 12$ Hz) reported elsewhere [32]. However, the observed difference in coupling constant is reasonable in that the coordinated Al(III) ion would tend to force the coordination sphere toward a planar conformation as in coordinated Schiff bases [33], and would polarize the ligand by its electron-withdrawal effect. Hemiacetaldimine formation is probably difficult to observe in other pyridoxal-catalyzed transamination systems because its resonance would be buried under the HOD peak. Because of the phenyl substituent in **6**, the 5′-CH_2 proton resonance apparently is shifted sufficiently to be observed.

As the reaction proceeded the resonances due to **6** were observed to grow in intensity with the exception of the α-proton. The rate at which this system passes through the αDI was followed by comparing the rate of deuteration of the α-proton with the growth of aldimine complex concentration, as measured by the 4′-CH resonance. Since the aldimine and αDI chemical shifts are not observed directly, these species must be present at concentrations too low for detection by NMR under the conditions employed. The resonances of the 2′-CH_3 protons of the ketimine Schiff base complexes grow very little with time compared to the loss of the α-proton, but this may or may not indicate extensive racemization relative to transamination.

The reluctance of this system to undergo transamination is also observed when treating benzoylformic acid (the keto-analog of phenylglycine) with pyridoxamine. After one hour some ketimine formation was observed; however, after 18 h 44 min at room temperature, 25.0 °C, virtually no further increase in ketimine concentration occurred, and no aldimine or transamination products were observed by NMR. It may be concluded, however, that the aldimine was formed after an extended time lapse, because its characteristic yellow color was observed.

Conclusions

Coordination by Al(III) sufficiently stabilizes the delocalized carbanion (αDI) formed by deprotonation of aldimines and ketimines to render it detectable by proton NMR in D_2O solution. Observation of the changes of αDI concentration with time in a transamination system approaching equilibrium in which aldimine predominates over ketimine demonstrates its function as an intermediate in the transamination pathway. Thus in the aldimine ⇌ ketimine system formed from pyridoxal–alanine, the αDI concentration lags behind the aldimine concentration and eventually reaches a steady state, with a concentration considerably lower than that of the aldimine. When the same equilibrium mixture is prepared from pyridoxamine and pyruvate, the αDI is formed initially in concentrations greater than those of the aldimine, and then decreases as aldimine concentration builds up, both species eventually reaching their equilibrium values, which are the same as those attained from the opposite direction. No αDI is formed from pyridoxal and α-aminoisobutyric acid, in accordance with the requirement for the α-proton of the amino acid moiety.

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