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Pyridoxal Methochloride Catalysis of the β-Elimination Reaction of Methyl 3-Chloroalaninate in Water

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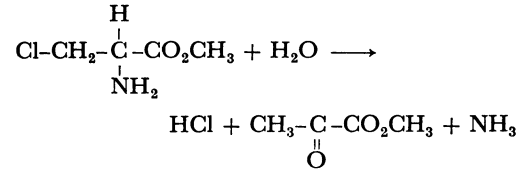
The β-elimination reaction of methyl 3-chloroalaninate has been investigated in the absence and presence of pyridoxal methochloride (**2**) or pyridoxal (**3**). Apparently, compound **2** was about twice as effective as **3** in the catalytic reaction that proceeds via a Schiff base of substrate and **2** or **3**. The pH-rate profiles for the catalytic reaction with **2** and **3** were analyzed in terms of ionization of the various Schiff base species present in solution. It was found that the reactivity of the Schiff base decreases as the ionization of the molecule progresses. The reaction mechanism and its relevance to the catalysis of pyridoxal phosphate-dependent enzymes are discussed.

Pyridoxal phosphate (PLP) is a coenzyme involved in the metabolism of amino acids in biological systems. These metabolic reactions include transamination, racemization and decarboxylation. They can be mimicked in model systems mostly in the presence of metal ions and at high temperature.[1] Through these biomimetic studies an insight was gained into the reaction mechanism of pyridoxal catalysis in enzymic systems. Thus, a Schiff base formed between pyridoxal and amino acid is always postulated as a key intermediate in all the reactions mentioned above. Nevertheless, much remains to be elucidated as to the catalytic mechanism of this and related vitamins, *e.g.*, the protonation state of a reactive Schiff base species. This particular problem happens because pyridoxal and the Schiff base derived therefrom can have various ionic forms in aqueous media that differ in number and/or the site of protonation.[2] We have been interested in the pyridoxal catalysis of the β-elimination reaction of amino acids in the absence of a metal ion.[3] 3-Chloroalanine is a substrate suitable for mechanistic studies, as this compound is known to undergo β-elimination spontaneously[4] and in the presence of pyridoxal.[5] In addition, an enzyme which promotes β-elimination of 3-chloro-D-alanine was recently found in *Pseudomonas putida* CR 1—1.[6] In this article, we have attempted a mechanistic study on the pyridoxal methochloride catalysis of the reaction of methyl 3-chloroalaninate (Eq. 1). This particular catalyst was chosen, because there are only two ionic forms of Schiff base for this compound (Scheme 1). This should facilitate the analysis of kinetic data on the catalytic β-elimination of the substrate.

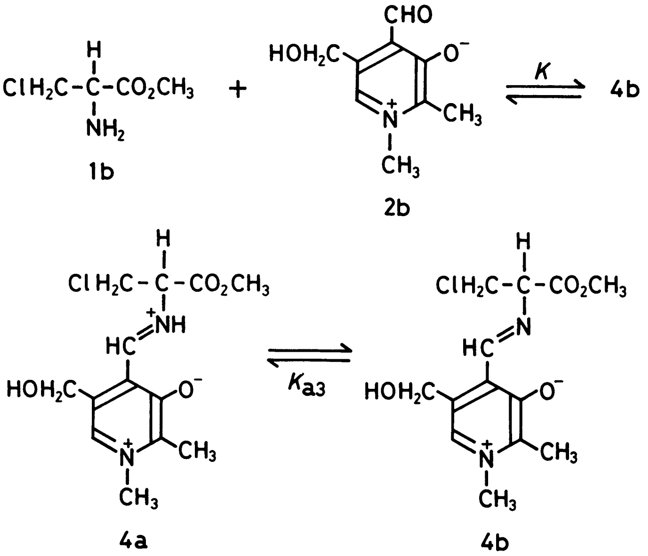


$$\mathrm{Cl{-}CH_2{-}\underset{NH_2}{\overset{H}{C}}{-}CO_2CH_3 + H_2O \longrightarrow HCl + CH_3{-}\underset{O}{\overset{}{\underset{\|}{C}}}{-}CO_2CH_3 + NH_3} \qquad (1)$$

Experimental

Materials. Pyridoxal hydrochloride was obtained from Wako Pure Chemical Ind. Pyridoxal methochloride (**2**) was prepared from pyridoxal (**3**) according to the literature.[7] Methyl L-3-chloroalaninate hydrochloride (**1**) was prepared from dimethyl L-cystinate and chlorine.[8] All other chemicals were obtained from commercial sources. Glass-distilled water was used in all experiments.

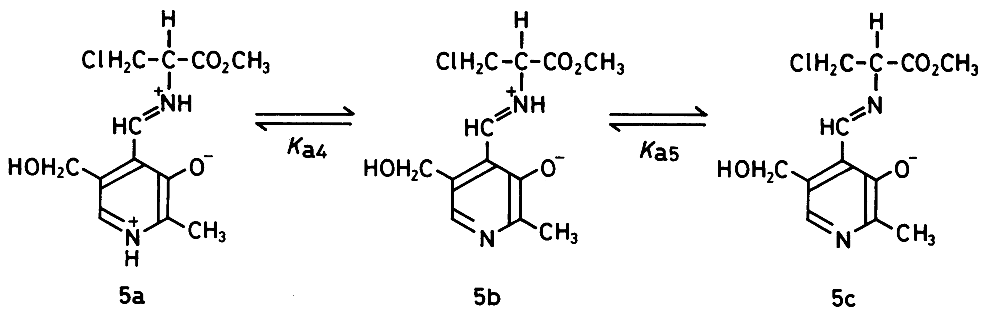
Apparatus. Electronic absorption spectra were taken on a Hitachi 124 spectrophotometer. The pH values of aqueous solutions were determined with a Toa pH meter HM-5A connected with a Toa combination electrode GS 195C. Computations were carried out on a Sord microcomputer 223 Mark II with a program written in the Basic.



Scheme 1.

Equilibrium Measurements. Since 3-chloroalanine is labile at ambient temperatures, equilibrium studies on the formation of a Schiff base of **2** were carried out with alanine. The pertinent equilibria are depicted in Scheme 1 (but note that the amino acid illustrated is **1** but not alanine). The amino acid, **2** and the Schiff base derived thereof exist over the pH range of 5 to 11 in two ionic forms.[9] The protonated species is designated by the symbol a, while the deprotonated species by b. The acid dissociation constants of amino acid, **2** and the Schiff base are referred to as K_{a1}, K_{a2}, and K_{a3}, respectively. The pK_{a2} for **2** was determined as 4.0 by a spectrophotometric titration. The protonated form of Schiff base (HSB or **4a**) has an absorption maximum at 414 nm and the deprotonated form (SB^- or **4b**) at 374 nm. To determine both the equilibrium constant for the Schiff base formation K, defined by Eq. 2, and pK_{a3} electronic absorption spectra were taken at pH values 6.00, 7.00, 8.00, and 9.00 by varying the concentrations of alanine (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 M) at 30.0 °C, μ=1.0 (KCl). Equilibrium was reached within a few minutes after mixing. The data were analyzed according

$$K = \frac{[\mathbf{4b}]}{[\mathbf{1b}]\,[\mathbf{2b}]} \qquad (2)$$



Scheme 2.

basically to the same method as that described previously.[10–12] Other pK_a values used in the calculation were 4.0 and 9.2 for **2** and alanine, respectively. The results are the following: K 5±1 M^{-1}†, pK_{a3} 8.0±0.2. The molar extinction coefficients of HSB and SB^- are 4000±1000 and 6000±1000 at 374 nm, and 8000±2000 and 1200±100 cm^{-1} M^{-1} at 414 nm, respectively. The corresponding equilibrium constant for the Schiff base of **3** and alanine was reported as 9 M^{-1}.[10]

Kinetics. All experiments were carried out at 25.0 °C, unless otherwise specified. The rate of the β-elimination reaction of **1** was determined by following the release of chloride ion by means of Volhard titration. The reaction mixture (10 ml) consisted of 0.010 M **1**, 0—0.020 M **2** or **3** in 0.10 M phosphate (pH 6—8.5) or borate buffer (pH>8.5). An aliquot (1 ml) was withdrawn from the reaction solution at appropriate time intervals, quickly transferred to a quenching solution (6 ml) containing 0.083 M silver nitrate and 1 M nitric acid and kept on ice for later titration. The excess silver ion was back-titrated with 0.1 M potassium thiocyanate in the presence of ferric ammonium sulfate as indicator. The amount of chloride ion at infinite time agreed well with the theoretical value. Data obeyed pseudo-first-order kinetics with usually a correlation coefficient 0.99 or greater.

Another reaction product, methyl pyruvate, was determined by the 2,4-dinitrophenylhydrazone method. The hydrazone was assayed spectrophotometrically at 530 nm (ε 10500),[13] after removing the insoluble hydrazone of **2** or **3** by filtration. The amount of pyruvate at infinite time agreed well with the theoretical value.

Data Processing. The catalytic rate constant k_2 of Eq. 7 was analyzed in terms of a difference in reactivity of the Schiff base species involved. As shown in Schemes 1 and 2, the Schiff bases of **2** and **3** are present in two or three ionic forms over the pH range studied.[9] Thus this idea is formulated as in Eqs. 3 and 4 for **2** and **3**, respectively. By combining Eqs. 2 and 3 one

$$k_2[\mathbf{1}][\mathbf{2}] = k_a[\mathbf{4a}] + k_b[\mathbf{4b}] \qquad (3)$$

$$k_2[\mathbf{1}][\mathbf{3}] = k'_a[\mathbf{5a}] + k'_b[\mathbf{5b}] + k'_c[\mathbf{5c}] \qquad (4)$$

obtains Eq. 5, where K_{pH} is the operational equilibrium constant defined by Eq. 6. Since the pK_{a2} for **2** is known, four

$$k_2 = \{k_a[H^+]+k_bK_{a3}\} \times \frac{K_{pH}}{[H^+]+K_{a3}} \qquad (5)$$

$$K_{pH} = \frac{\left\{1+\frac{[H^+]}{K_{a3}}\right\}K}{\left\{1+\frac{[H^+]}{K_{a1}}\right\}\left\{1+\frac{[H^+]}{K_{a2}}\right\}} \qquad (6)$$

unknown parameters, K_{a1}, K_{a3}, k_a, and k_b remain to be determined. They were optimized by essentially the same procedures as those described for the analysis of spectroscopic data. It should be noted here that the Schiff base formation constants K used in the calculation were those for alanine, because we were unable to determine the corresponding values for **1** precisely. Although the equilibrium constants for alanine and **1** may not be exactly identical, it would be reasonable to assume that the relative magnitudes of the constants for **2** and **3** do not differ significantly between alanine and **1**. We have confirmed that as long as this assumption holds, the main conclusions drawn by this data processing do not change appreciably.

Results

The β-elimination reaction of **1** in buffered aqueous media obeyed a pseudo-first-order rate law with respect to the substrate both in the absence and presence of catalyst **2** or **3**. It was found that the observed first-order rate constants are dependent linearly on the catalyst concentration over a 0—0.02 M range. The k_1 term of Eq. 7 represents the spontaneous reaction of the

$$k_{obsd} = k_1 + k_2[\mathbf{2}\text{ or }\mathbf{3}] \qquad (7)$$

substrate. The logarithm of k_1 increases slightly with pH, but the slope of a straight line (0.12) fitted to data points is far from unity (Fig. 1), suggesting that the specific base catalysis is not working over the pH range studied. A similar insensitivity of rates to pH was reported in the β-elimination of *O*-phosphoserine over a pH range of 7 to 14.[14] It should be noted that the linear analysis of kinetic data in Fig. 1 is an approximation, as the rate increase at pH 7—8 could reflect the acid dissociation of **1a** (protonated form) to **1b**. If the data are analyzed by this scheme, **1b** is about twice as reactive as **1a**. The k_2 term refers to the catalytic reaction, proceeding via a Schiff base of the substrate

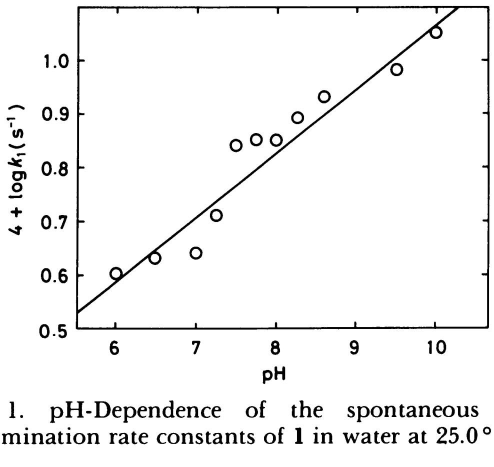


Fig. 1. pH-Dependence of the spontaneous β-elimination rate constants of **1** in water at 25.0 °C.

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† 1M=1 mol dm^{-3}.

Table 1. Specific rate constants of individual Schiff base species in the β-elimination of **1** with **2** or **3** in water at 25.0 °C

k_a	k_b	k'_a	k'_b	k'_c
		s^{-1}		
0.11	0.017	0.070	0.010	0.004

a) For definition of the rate constants refer to Eqs. 3 and 4 and Schemes 1 and 2.

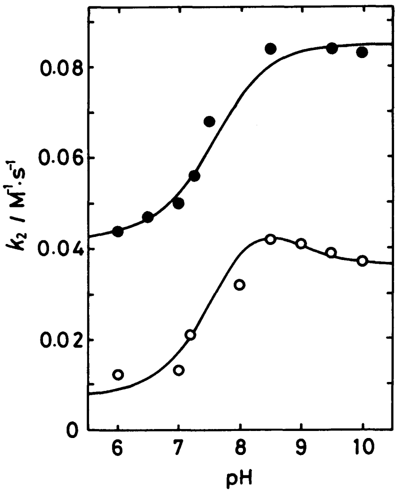


Fig. 2. pH-Rate profiles for the β-elimination of the Schiff base of **1** with **2** (●) and **3** (○) at 25.0°C. The theoretical curves were drawn based on the rate constants given in Table 1 and the following pK_a values: pK_{a1} 7.6; pK_{a2} 4.0; pK_{a3} 6.5; pK_{a4} 5.9; pK_{a5} 8.5; pK_a of **3** 4.23 and 8.70 (V. R. Williams and J. B. Nielands, *Arch. Biochem. Biophys.*,**53**, 56 (1954)).

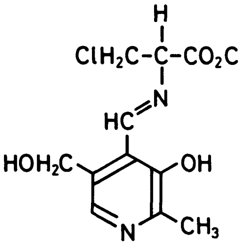
with **2** or **3**. The treatment according to Eq. 7 is warranted, since the Schiff base formation is not extensive under the present conditions. The pH dependence of k_2 is shown in Fig. 2 for both **2** and **3**. Compound **2** is apparently twice as effective as **3** at any pH value studied.

The two pH-rate profiles for the β-elimination of the Schiff base of **1** were interpreted in terms of a difference in reactivity of the various species of Schiff base (see Experimental section). The Schiff base of **2** is present in two ionic forms, **4a** and **4b** (Scheme 1), and the experimental data are explicable by the sigmoidal curve generated with the aid of the parameters listed in Table 1. It is manifest that the protonated species (**4a**) is 6.5 times more reactive than **4b**. There are three ionic species, **5a**, **5b**, and **5c** (Scheme 2), in the Schiff base of **3** in the pH region studied, and the skewed bell-shaped curve created by the parameters listed in Table 1 fits neatly the data points. The analysis clearly reveals that the specific rate constant decreases with the degree of deprotonation on the Schiff base molecule, *i.e.*, **5a**>**5b**>**5c**.

Activation parameters for the spontaneous and **2**-catalyzed β-elimination reaction of **1** were determined at pH 10. The Schiff base of **1** and **2** exists solely in the deprotonated form (**4b**) and the parameters obtained represent those for the SB^- species (Table 2). The reaction is characterized by the large negative entropy of activation and this is compatible with the bimolecular character of β-elimination reactions.[3] The smaller enthalpy of activation for the catalytic reaction may be associated with the labilization of the substrate's α-hydrogen through formation of a Schiff base with **2**.

Discussion

The purpose of the present investigation was twofold. A mechanistic study of PLP-dependent reactions has often been complicated, because the active Schiff base exists in a variety of ionic forms that differ in reactivity (*e.g.*, see Scheme 2). If one takes into account prototropic equilibrium between, for example, **5b** and **5d**, which could happen in a medium of low dielectric constant,[2] the number of the active Schiff bases further increases. In contrast, the number of Schiff base species derived from an *N*-substituted pyridoxal like **2** is small (Scheme 1). This should ease the analysis of kinetic data in a Schiff base reaction, as has been proven above. Moreover, the presence of a positive charge as a result of the *N*-substitution on the pyridoxal molecule should increase the electron-withdrawing ability of the catalyst, leading to labilization of the α-hydrogen of the Schiff base.[15] This idea has been supported by the finding that **2** is more effective both apparently (Fig. 2) and in terms of specific rate constants than **3** in the elimination reaction of **1** (Table 1).



5d

The results given in Table 1 demonstrate that the Schiff bases of **2** and **3** are more reactive when they have more positive charges on them. These data agree with the above argument and may also be reasonable from a

Table 2. Activation parameters for the spontaneous and **2**-catalyzed β-elimination of **1** in water at pH 10

	Rate constant	Temperature/°C			E_a	$\Delta H^{\neq}$ a)	$\Delta S^{\neq}$
		15	25	30	kcal mol^{-1} b)	kcal mol^{-1} b)	e.u.
Spontaneous	$k_1 \times 10^3/s^{-1}$	0.84	1.29	1.42	6.3	5.8	−53
Catalytic	$k_2 \times 10/s^{-1}\ M^{-1}$	0.64	0.83	1.01	5.3	4.7	−48

a) Values at 25.0 °C. b) 1 cal=4.184 J.

mechanistic point of view, as PLP serves basically as an electron sink in its catalysis.$^{16)}$ It may be fair, however, to mention the kinetic equivalence of the reaction, since others dealing with similar data reached a somewhat different conclusion.$^{5)}$ For example, the reaction of **4b** is equivalent kinetically to the reaction of **4a** with hydroxide ion (Eq. 8), where K_w stands for the

$$k_b[\mathbf{4b}] = k_b'' \frac{K_{a3}}{K_w} [\mathbf{4a}][\mathrm{OH}^-] \tag{8}$$

ionization product of water. Although it is not possible to distinguish rigorously between these two mechanisms at present, we favor the k_b pathway for the following reason. It is obvious from the spectroscopic data that the Schiff base of **2** exists in two ionic forms, **4a** and **4b**. If one assumes that the k_b pathway is actually the k_b'' pathway, *i.e.*, the reaction of **4a** with OH^-, one could naturally expect a similar reaction for **4b**. Since the concentration of **4b** is over a thousand times higher than that of **4a** at pH 10, this could have brought about a pH-dependent rate increase in the alkaline region, unless **4a** is over a thousand times more reactive than **4b** in the reaction with OH^-. Hence, the lack of such a tendency at high pH values supports the contention that the k_b pathway is the more likely one.

It is noticeable that the catalytic β-elimination is not subject to the hydroxide ion catalysis. This may be understood, since the spontaneous reaction is little affected by the hydroxide ion concentration and in the catalytic reaction the electron sink labilizes the α-hydrogen so that a strong base such as hydroxide ion may not be needed for its abstraction. We suppose that water is the base responsible for the abstraction of α-hydrogen. This may raise a question about the occurrence of general base catalysis in the Schiff base reaction. An increase in phosphate buffer concentration from 0.10 to 1.0 M at pH 8 and at different catalyst concentraions gave rise to a modest increase (15% at maximum) in the observed rate, indicating that at least the buffer components used in these experiments do not affect the rates significantly. Finally, the relevance of the present data to the enzymatic catalysis is mentioned. A number of PLP-dependent enzymes including 3-chloro-D-alanine chloride-lyase show an absorption maximum at ≈420 nm.$^{17)}$ Unambiguous assignment of this band is obscured, since **4a**, **5a** and **5b** provide similar spectra.$^{10)}$ In light of the fact that **4a** and **5a** show the highest reactivity in the present reaction, the enzyme-bound PLP may carry a proton on the pyridine nitrogen.$^{15)}$ This could explain in part a higher reactivity of the coenzyme in enzymatic systems.

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