Intermediate Species Absorbing in the 500-nm Region in Al(III) Catalyzed Nonenzymatic Transamination

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Course of the formation of a quinonoid species absorbing in the 500-nm region, which should serve as a model for the key intermediate in reactions catalyzed by pyridoxal phosphate enzymes, was studied in the reaction of pyridoxal, ethyl L-alaninate and Al(III) in methanol by means of UV–visible spectroscopy, chromatography with a multi-UV detector, optical rotation and ^{1}H-NMR. The species was chromatographically separated in the form of the Al(III) chelate and its quantitative transformation to the aldimine chelate was observed. The formation of the species in the transamination reaction of pyridoxamine, ethyl pyruvate and Al(III) was also studied.

Key words pyridoxal; quinonoid intermediate; Al(III) chelate; ethyl alaninate; aldimine

Pyridoxal phosphate (PLP) is a ubiquitous cofactor of enzymes whose functions are amino acid metabolisms.[1] A key step in the action of almost all PLP enzymes is the formation of a quinonoid species, in which the α-carbon in a Schiff base (aldimine) formed from PLP and amino acid is deprotonated. Several enzymes have been reported to exhibit an intense and transient absorption band in the 500-nm region of the spectrum which has been ascribed to the quinonoid species,[2] though the band is unobservable in most enzymes. Chart 1 shows the generally accepted mechanism of transamination catalyzed by PLP dependent enzymes.

Metal ion mediated nonenzymatic reactions of pyridoxal (PL) and amino acid derivatives and those of pyridoxamine (PM) and α-keto acid derivatives have been proved to be useful tools for the mechanistic investigation of pyridoxal functions.[3] We reported previously that in methanolic solutions PM and ester of an α-keto acid with Al(III) gave an intense and transient absorption band at the 500-nm region and the species should serve as a model of the enzymatic intermediate.[4] For further characterization of the species, we have tried the liquid chromatographic separation of the species involved in the reactions.

Experimental

Procedures The following reactions in methanol were studied: 1. PL+ethyl L-alaninate (AlaOEt); 2. PL+AlaOEt+Al(III) (aluminum perchlorate); 3. PM+ethyl pyruvate; 4. PM+ethyl pyruvate+Al(III). Reactions in which AlaOEt and ethyl pyruvate were replaced by L-alanine (Ala) and sodium pyruvate, respectively, were also examined.

Calculated volumes of methanol solutions of the reactants were mixed in a predetermined order and kept in a bath thermostated at 25 °C in the dark. Detailed procedures for preparation of the solutions were described previously.[4] The spectral changes were recorded on a Shimadzu UV–240 UV-visible recording spectrophotometer. Samples were withdrawn at intervals and injected into a HPLC equipped with a multi-wavelength UV-visible detector.

The optical rotation was recorded with a DIP-140 Digital Polarimeter

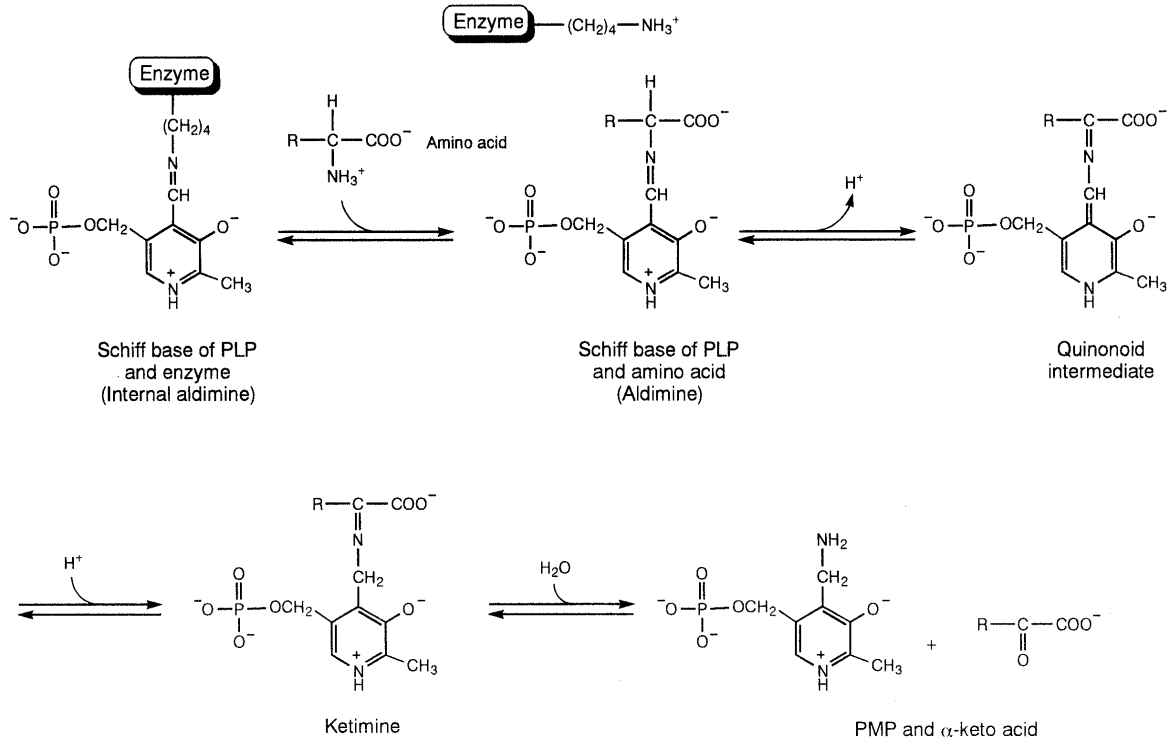

Chart 1. Reaction Mechanism of Transamination Catalyzed by PLP Dependent Enzymes

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(Japan Spectroscopic Co., Ltd.). The rotation was expressed as measured by D-ray of Na. ^{1}H-NMR spectra were recorded with a JEOL JNM-α500 spectrometer. Chemical shifts are expressed in ppm on the δ scale from tetramethylsilane as the internal standard.

HPLC Conditions Pump: Shimadzu LC-6A; Detector: Hitachi L-4500 Diode Array; Column: Hitachi gel #3056 Silica ODS (4 mm i.d. ×250 mm); Eluent: 0.4 mM $HClO_4$ in CH_3OH; Flow rate: 1.0 ml/min.

Chemicals PL hydrochloride, PM dihydrochloride, and Ala ethyl ester hydrochloride (AlaOEt) were purchased from Sigma Chemical Co. and were neutralized with alkaline methanol immediately before use. HPLC grade of methanol was purchased from Wako Pure Chemical Industries, Ltd. The other chemicals were of reagent grade, and obtained commercially.

Results

Under the HPLC conditions, PL showed a single peak at 2.8 min, with an absorption band at 290 nm. In methanol solution of PL (0.5 mM) and AlaOEt (0.5 mM) (solution 1), a Schiff base (aldimine, *N*-pyridoxylidenealanine ethyl ester) with absorption band at 335 nm and at 250 nm and a less intense band at 420 nm was gradually formed. The chromatogram of the solution 19 h after the mixing indicated the presence of the aldimine with the retention time (t_R) of 3.0 min and a small amount of PL. Similar spectra and chromatograms were obtained in methanol solution of PL and Ala, though the formation of the aldimine (*N*-pyridoxylidenealanine) was faster and more complete and its band at 420 nm assignable to ketoenamine species[3b,3d,3f,5] was more intense.

PL and AlaOEt were mixed in methanol and equilibrated. Addition of Al(III) ion to the solution 3 h after the mixing (solution 2) gave rise to an intense absorption at 490 nm, which decreased gradually and disappeared in several hours.[4b] In a mixture containing 0.5 mM each of PL, AlaOEt and Al(III) perchlorate,[6] absorbance at 490 nm attained maximum at 15 min. In the chromatogram obtained by injecting the mixture at this stage, a fraction having an intense 490-nm absorption was separated at t_R 6.0 min from those of PL and the aldimine. The spectrum of the fraction had no significant absorption at 380—400 nm. The fraction is assumed to contain the short-lived Al(III) chelate of the quinonoid species and a negligible amount of the aldimine species.

The separated fraction (solution 2a) showed a gradual spectral change on standing as shown in Fig. 1. The 490-nm band disappeared forming a set of clear isosbestic points and a new band appeared at around 366 nm. The chromatogram of 2a after the disappearance of the 490-nm absorption had a fraction with an absorption at 366 nm, which can be assigned to the Al(III) chelate of the aldimine. The results showed that the separated quinonoid species was metastable in the form of Al(III) chelate, which was gradually converted to Al(III) chelate of the aldimine of PL and AlaOEt. The addition of sodium ethylenediaminetetraacetate (EDTA) to the 490-nm absorbing fraction resulted in the instantaneous disappearance of the absorption and formation of the spectrum of the aldimine, which indicated that the unchelated form of the quinonoid species is too unstable to be observed.

The chromatogram obtained by the injection of solution 2 after the disappearance of the 490-nm absorption had the peaks assignable to PL, the aldimine and its Al(III) chelate. Replacement of AlaOEt by Ala in solution 2 did not appreciably form the band at the visible region and the band assignable to Al(III) chelate of the aldimine was formed immediately after the addition of Al(III).[4a,b]

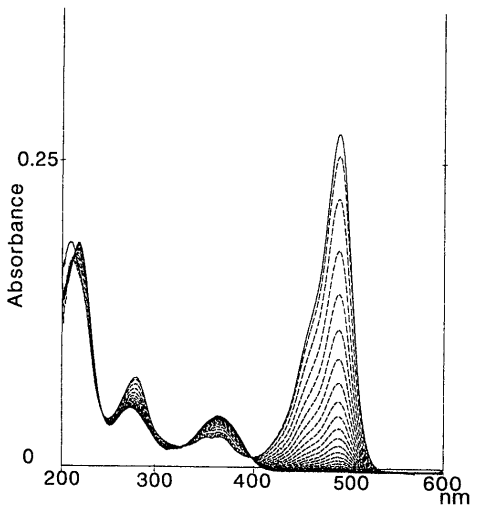

Fig. 1. Spectral Changes with Time of Separated 490-nm Absorbing Species in the Reaction of PL, AlaOEt and Al(III) in Methanol

Solid line absorbing 490 nm, immediately after the separation; dotted lines, 4.2, 10, 20, 30, 40, 50 min, and 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5 h after the separation in the decreasing order of the 490-nm absorption; solid line without the absorption, 8 h after the separation.

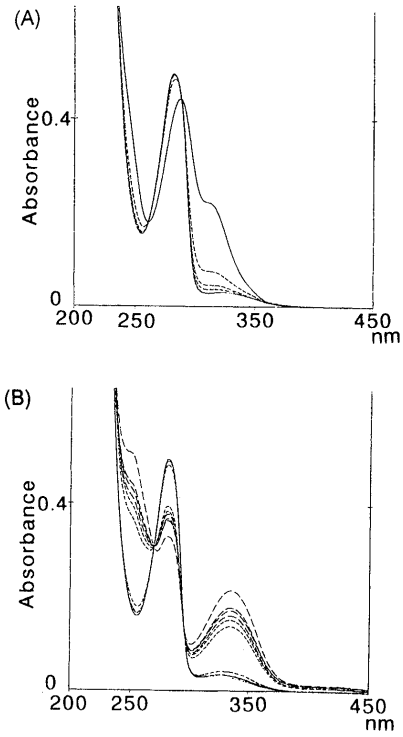

Fig. 2. Spectral Changes with Time of a Mixture of PM and Ethyl Pyruvate in Methanol

Times after the mixing. A. Solid line with a shoulder at 310 nm, immediately after the mixing; dotted lines, 20, 30, 40 and 50 min, in the decreasing order of the shoulder; solid line with the highest absorption at 285 nm, 70 min. B. Solid line with the highest absorption at 285 nm, 80 min; dotted lines, 100, 130 min, and 22, 24, 26, 28, 30, 47 h in the increasing order of the 335-nm absorption.

A methanol solution of PM and ethyl pyruvate (solution 3) showed two steps of spectral changes as shown in Fig. 2. The first step was completed in 2 h and showed the formation of the ketimine[3,4] (Fig. 2A). The retention times of PM and the ketimine were 9.3 and 2.8 min, respectively. The second step was very gradual and almost completed in several days (Fig. 2B). A peak assignable to the aldimine of PL and AlaOEt was in the chromatogram 8 h after the mixing of PM and

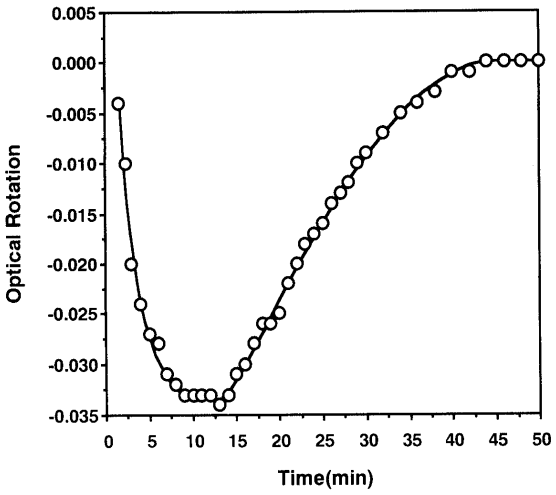

Fig. 3. Optical Rotation in the Reaction of PL (0.02 M) and AlaOEt (0.02 M) in Methanol

The numbers on the abscissa indicate time after the initiation of the reaction.

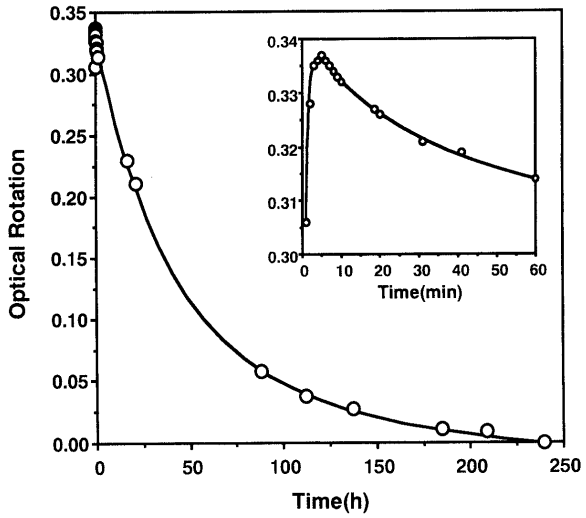

Fig. 4. Optical Rotation in the Reaction of PL (0.02 M) and Ala (0.02 M) in Methanol

ethyl pyruvate. The spectral changes and chromatograms showed the very gradual interconversion of the ketimine to the aldimine in their ester forms in the absence of polyvalent metal ions. With a methanol solution of PM and sodium pyruvate, spectral and chromatographic results showed gradual formation of the ketimine, which was not converted to the aldimine under the same conditions even after several days.

Solutions 4a and 4b were prepared by addition of Al(III) to solution 3, 1 h and 24 h after the mixing to PM and ethyl pyruvate, respectively. The chromatogram of 4a immediately after the Al(III) addition showed the presence of Al(III) chelates of the ketimine and the 490-nm species. The spectral change of the mixture indicated the Al(III) chelate-mediated isomerization of the ketimine to the aldimine *via* the quinonoid intermediate, as reported previously in another α-keto acid ester.[4a] The spectral changes and the chromatograms of 4b were essentially the same as those of solution 2.

Changes of the optical rotation with time were measured for the reaction PL (0.02 M) and AlaOEt (0.02 M), and the results are shown in Fig. 3. Methanol solution of AlaOEt (0.02 M) showed optical rotation of $+0.01°$. On addition of an equimolar PL, the optical rotation turned rapidly to negative, reached the minimum value after 13 min, and then gradually approached zero asymptotically. During the course of the rotational change, the absorption spectra indicated gradual formation of the aldimine. The rapid change to negative value at the first phase was probably due to the formation of the aldimine of AlaOEt, whose specific rotation would be of a large negative value. The slow change to zero at the second phase should indicate the racemization of the optically active aldimine. In the presence of Al(III), rate of the racemization was increased.

In the reaction of Ala with PL, the rotation initially showed large positive values, reached a maximum value in several min and decreased very slowly, which is shown in Fig. 4. The racemization was much slower than that of AlaOEt, though the aldimine formation measured by the spectral changes was faster.

^{1}H-NMR spectra of tetradeuteromethanol (CD_3OD) solution of PL and AlaOEt showed a quartet signal at 4.36 ppm and a doublet signal at 1.56 ppm, which are assigned to the α-proton and β-protons of alanine ester moiety of the aldimine, respectively. The 4.36-ppm signal disappeared and the 1.56-ppm signal became singlet in the time scale comparable to the racemization measured in the optical rotation.[7]

Discussion

Chart 2 illustrates the proposed mechanism. In the absence of the metal ion, the reaction of equimolar amounts of PL and AlaOEt reached equilibrium containing a large amount of the aldimine and small amounts of the two reactants. Though the unchelated quinonoid species was not detectable by absorption spectra, its presence was clearly shown by the results of the optical rotation, ^{1}H-NMR and Al(III) addition. The unchelated quinonoid species should be present in a very small amount in the equilibrium with the unchelated aldimine. The reaction of PM and ethyl pyruvate initially formed the ketimine. In the second step, a small portion of the accumulated ketimine was converted to the unchelated quinonoid species, which in turn transformed into the aldimine. Thus, both reactions reached the same equilibrium after several days.

When Al(III) was added to the equilibrium mixture, the quinonoid species most rapidly formed the metal chelate, which was gradually transformed into the metal chelate of the aldimine. Since the formation of unchelated quinonoid from the aldimine is a rapid step, we were able to observe the accumulation of the 490-nm absorbing Al(III) chelate of the quinonoid. Direct formation of Al(III) chelate from the aldimine of AlaOEt must be a slow step.

In the reaction of PL and Ala, the amount of the unchelated quinonoid species in the equilibrium with the aldimine should be trace and far less than in that of PL and AlaOEt. This was shown by the slower racemization step and the absence of the 500-nm species on addition of Al(III).

Conclusion

Though a few researchers have shown spectrometrically the presence of the quinonoid species in enzymatic and nonenzymatic reactions, its separation has not been reported so far. We have shown that the unchelated quinonoid species

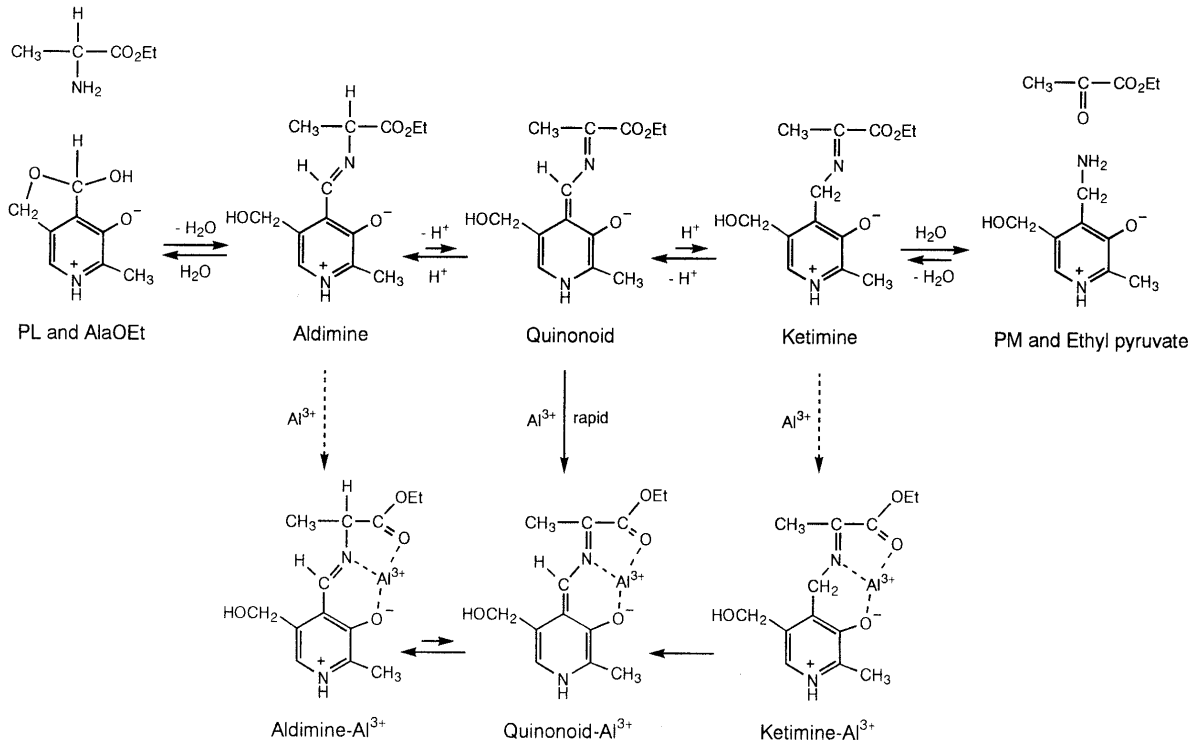

Chart 2. Nonenzymatic Transamination Involving the Quinonoid Intermediate

Dotted arrows indicate slow steps.

from PL and AlaOEt is present in a small amount in the equilibrium with the aldimine. The species is made metastable in the form of Al(III) chelate and the chelate was separated chromatographically and was spontaneously and quantitatively transformed to the aldimine chelate.

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References and Notes

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6) Replacement of Al(III) perchlorate by Al(III) nitrate or chloride in the reaction did not essentially change the spectral features or the kinetics. Changes in the concentrations and their ratios of the three reactants did affect the intensity and the lifetime of the 490-nm species, though the general spectral features were similar. The kinetic studies of the formation of the species will be reported elsewhere.

7) ^{1}H-NMR signals and their assignments to the species contained in the equilibrium mixture will be reported in near future with the NMR data of other reaction systems.