Species Absorbing in the 500-nm Region in the Reactions of Pyridoxamine with Pyrroloquinoline Quinone and Phenathrolinequinones

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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 formed in the reactions of pyridoxamine (PM) with pyrroloquinoline quinone (PQQ), 1,7-phenathroline-5,6-quinone and 4,7-phenathroline-5,6-quinone in alkaline methanol at 25 °C. The band at around 500 nm appeared gradually and its intensity reached a maximum about 6 h after initiation of the reaction, then decreased gradually and disappeared. 1,10-Phenathroline-5,6-quinone which lacks pyridine nitrogen peri to the *o*-quinone carbonyl groups did not react with primary amines under the conditions used. Crystalline product was prepared from PM and PQQ. Absorption spectra of its methanol and water solutions were similar to the spectrum formed from the reaction of PM and PQQ with 500 nm band assigned to the quinonoid. The results of FAB mass were consistent with the formula of the ketimine and the quinonoid formed from PM and PQQ.

Key words pyridoxamine; quinonoid intermediate; pyrroloquinoline quinone; 1,7-phenathroline-5,6-quinone; 4,7-phenathroline-5,6-quinone; 1,10-phenathroline-5,6-quinone

Pyridoxal 5-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 PL functions.[3] We found that in methanol solutions PM and ester of an α-keto acid with Al(III) gave an intense and transient absorption band in the 500-nm region and the species should serve as a model of the enzymatic intermediate.[4] We earlier showed[4h] the chromatographic separation of the species in the form of the Al(III) chelate and its quantitative transformation to the aldimine chelate.

Pyrroloquinoline quinone (PQQ) is a coenzyme discovered in several bacterial dehydrogenases.[5] Ohshiro, Itoh and coworkers[6] reported that the oxidative deamination of some amino acids with PQQ takes place catalytically in the presence of cetyltrimethylammonium bromide micelles under mild conditions. Eckert and Bruice[7] studied the mechanism of amine oxidation by *o*-quinones including PQQ. Churchich[8] reported that PQQ catalyzes the nonenzymatic conversion of PM and pyridoxamine 5-phosphate (PMP) into PL and PLP, respectively, in aqueous Tris–HCl buffer (pH 8.2) in the absence of micelles and proteins. In these studies, the formation of ketimines from the amines and 5-quinone carbonyl group of PQQ and the related *o*-quinones and subsequent isomerization to quinonoid intermediates were assumed as the initial part of the reaction mechanisms. The quinonoid species from PM should absorb in the visible region. However, species with visible absorption from the *o*-quinones has not been described in the literature. These urged us to investigate the reactions of PQQ and related substances (Chart 2) with PM in methanol.

Experimental

Chemicals PM dihydrochloride (PM·2HCl) was purchased from Sigma Chemical Co. PQQ was the product of Ube Industries, Ltd. 1,7-Phenathroline-5,6-quinone (1,7-PQ), 4,7-phenathroline-5,6-quinone (4,7-PQ) and 1,10-phenathroline-5,6-quinone (1,10-PQ) were prepared in this laboratory by the methods described in the cited references[9] with slight modifications. The other chemicals were of reagent grade and obtained commercially.

Procedures for Spectral Studies in Solution The reactions of PM with PQQ and phenathroline-5,6-quinones in methanol were studied in the presence of various concentrations of HCl or NaOH. Phenathrolinequinones studied were 1,7-PQ, 4,7-PQ and 1,10-PQ.

Calculated volumes of methanol solutions of the reactants were mixed in a predetermined order. A portion of the solution was transferred to a glass-stoppered 10-mm silica cell, sealed, kept in a air-bath thermostated at 25 °C in the dark and submitted to absorption measurement at predetermined intervals. The spectral changes were recorded on a Shimadzu UV-260 UV-visible recording spectrophotometer.

Preparation of Crystalline Product PM·2HCl (59 mg, 0.245 mmol) was suspended in methanol (20 ml), neutralized by addition of NaOH, and added to PQQ (80 mg, 0.242 mmol) solubilized in methanol (40 ml). After stirring overnight, the precipitate was filtered and washed with a small amount of methanol. The crystalline product was insoluble in most organic solvents, sparingly soluble in H_2O and methanol and did not show a melting point up to 300 °C. IR spectra were taken with a JASCO FT/IR-5300. FAB mass spectra were taken by Akihiko Kusai, JEOL Co., with a JMS-SX102A.

Results and Discussion

Spectral Studies in Solution Figure 1 shows changes of absorption spectra of a methanol solution (solution A), which contained 1.0×10^{-4} M PQQ, 1.0×10^{-4} M PM·2HCl and 1.0×10^{-3} M NaOH. In this slightly alkaline solution, a new absorption peak appeared at 516 nm with a shoulder at around 487 nm. Its intensity reached a maximum about 6 h after initiation of the reaction, then, the band decreased gradually and disappeared. Its disappearance was accelerated by contact with air.

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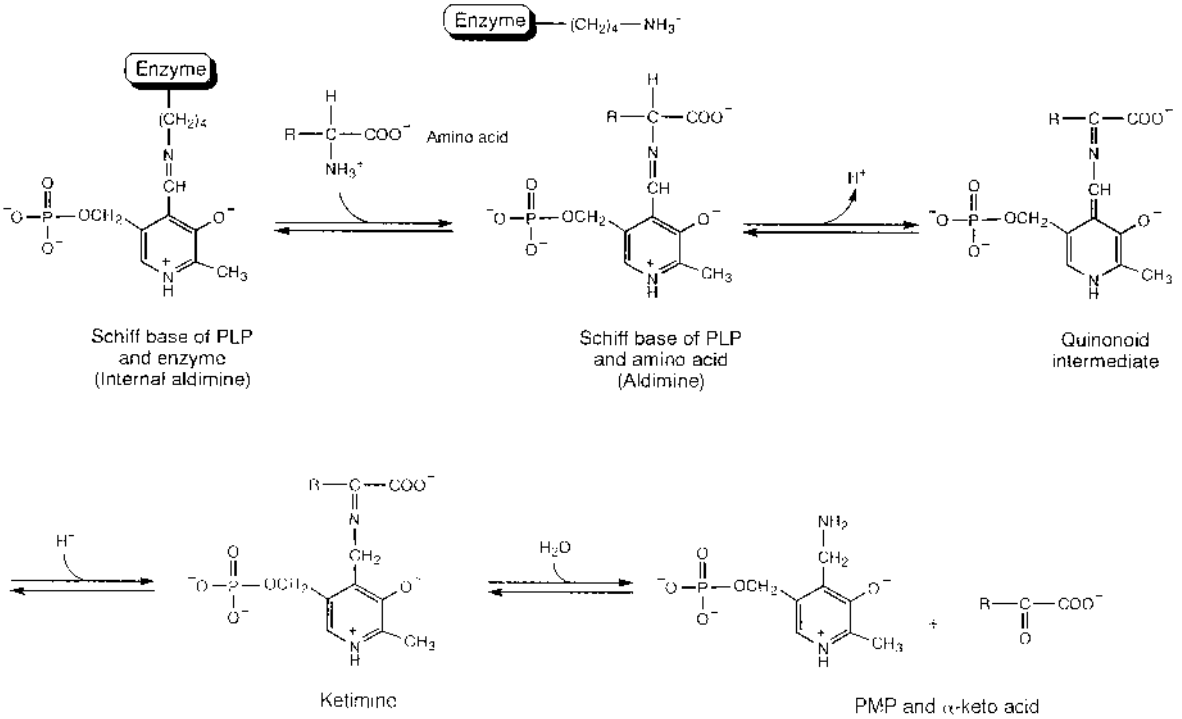

Chart 1. Reaction Mechanism of Transamination Catalyzed by Pyridoxal Phosphate Dependent Enzymes

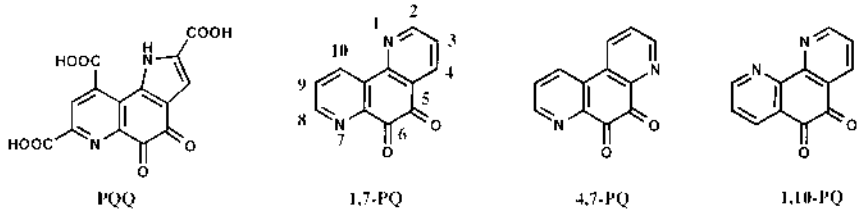

Chart 2. Structures of Quinones

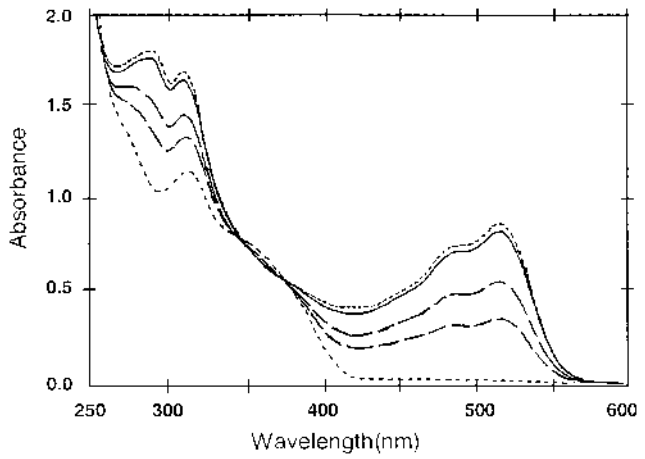

Fig. 1. Spectral Changes with Time of a Methanol Solution Containing 1×10^{-4} M PQQ, 1×10^{-4} M PM·2HCl and 1×10^{-3} M NaOH

Periods after mixing: immediately after the mixing and 0.5, 1.0, 4.0, 6.0 h afterward, in the increasing order of the absorption at 516 nm.

Solutions containing NaOH in the concentration range of 1.0×10^{-4} to 5.0×10^{-3} M were examined and solution A showed the most intense visible absorption. With a decrease of NaOH concentration, the intensity of the band decreased with long wavelength shifts of the peak to 539 nm and of the shoulder to 505 nm. In a solution of 1.0×10^{-4} M NaOH, absorbance at 500 nm at its maximum was one third that of solution A. No spectral change was observed under acidic conditions. The increase of NaOH concentration over 2.0×10^{-3} M resulted in the split of the band into two peaks and decreased intensity at the longer wavelength peak.

The band has the same spectral characteristics as those observed previously in Al(III) catalyzed pyridoxal catalysis[4b,4h] and is assigned to the quinonoid species derived from PQQ and PM. It can be concluded from the results and considerations described previously[4d] that the visible band is formed by overlapping the absorptions of the quinonoid species with protonated pyridine nitrogen at PM moiety (**1**) and that with unprotonated pyridine nitrogen (**2**). With a decrease of alkali concentration, the absorption due to **1** at around 540 nm became relatively intense, whereas that due to **2** at around 480 nm increased with an increase of alkali concentration.

Spectral changes obtained by the replacement of PQQ by 1,7-PQ or 4,7-PQ in solution A were almost identical with those of solution A in the visible region but significantly different from those in the UV region. The results showed that quinonoid species were formed from PM with 1,7-PQ or 4,7-PQ and the three kinds of quinonoids should have a common

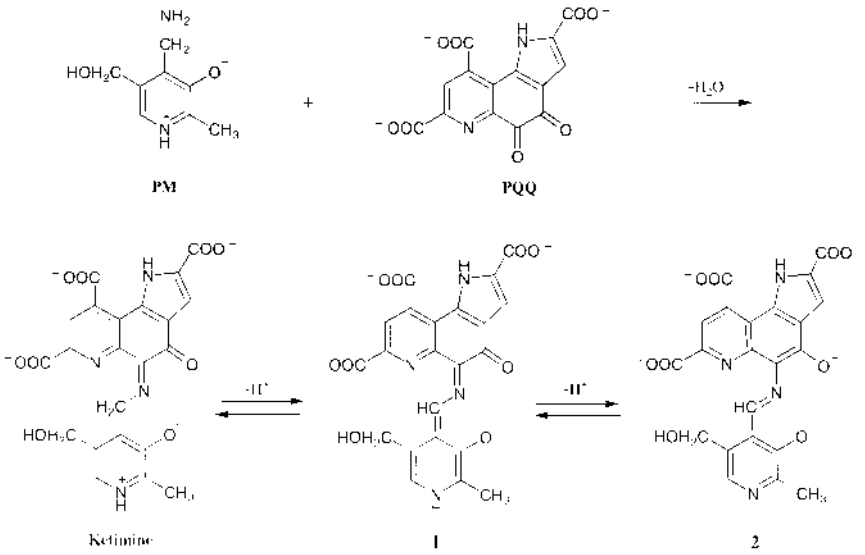

Chart 3. Reaction of PQQ and PM

chromophore responsible for the visible absorption. PQQ and the three phenathrolinequinones do not have absorption in the visible region and their spectra in the UV region are different. The replacement of PQQ by 1,10-PQ in solution A neither formed any visible absorption nor showed any spectral change in the UV region with time. It can be concluded that 1,10-PQ did not react with PM under the conditions used.

An increase in absorption at around 320 nm with time is seen in Fig. 1, with a decrease of 370-nm absorption of PQQ. We reported the increased absorption in the 320-nm region in the reactions of PM with α-keto acids or their derivatives and ascribed the absorption to ketimines.[4] Similar increase in absorption at 320 nm was also observed in the reactions of PM with 1,7-PQ and 4,7-PQ but not in that with 1,10-PQ. The absorption in the region may indicate the formation of the ketimine species formed from PM and quinones.

A methanol solution of 1.0×10^{-4} M PQQ and 1.0×10^{-3} M cyclohexylamine (solution B) showed very gradual spectral changes with time, including the decrease of 370-nm absorption of PQQ and the increase of absorption at around 325 nm. There appeared no visible absorption when the solution was allowed to stand. Similar spectral changes were observed by the replacement of cyclohexylamine in solution B with benzylamine but not with *N*-methylcyclohexylamine. The replacements of PQQ by 1,7-PQ and 4,7-PQ in solution B showed analogous spectral changes. These changes may reflect reactions of the quinones and primary amines in methanol, possibly formation of ketimines but not that of quinonoids. No spectral change was observed in methanolic mixtures of 1,10-PQ with the primary and secondary amines.

The spectral changes described above showed that in the reaction with PQQ, 1,7-PQ and 4,7-PQ, PM formed the ketimine, which was transformed to the quinonoid, and cyclohexylamine formed the ketimine, which was not transformed to the quinonoid. 1,10-PQ did not undergo similar reactions with the primary amines. Eckert and Bruice[7] reported that pyridine nitrogens peri to the *o*-quinone carbonyl groups increased the equilibrium constants for addition of H_2O and methanol to the latter in the order 4,7-PQ>1,7-PQ>1,10-PQ. It was assumed from the spectra in methanol solutions that considerable portions of 4,7-PQ and 1,7-PQ were present in the form of methanol adducts at the carbonyl, whereas 1,10-PQ was present in the unsolvated form. The reactive property of the quinone carbonyl of the phenanthrolinequinones may govern the reactions with the amines in the present study.

Crystalline Product from PM and PQQ The crystalline product from PM and PQQ was prepared as described in the experimental section. Its IR spectrum was similar to that of PQQ, but the intense band at 1645 cm^{-1} assigned to 5-carbonyl stretching vibration of PQQ had disappeared. This may indicate imine formation at this site in the product. Absorption spectra of freshly solubilized solutions of methanol and water were quite similar to that with maximum intensity at the 500-nm band shown in Fig. 1. Solubilities of the product in various solvents were examined, but were too small to give reliable ^{1}H-NMR data of the solutions.

FAB mass spectra were measured in triethanolamine (TEA) matrix. The results were (positive mode) *m/z* 481 $[M+H]^+$ and *m/z* 630 $[M+TEA+H]^+$, (negative mode) *m/z* 479 $[M-H]^-$ and *m/z* 628 $[M+TEA-H]^-$. These indicated that the molecular weight of the product was 480. Refined mass measurement at the *m/z* 479 (negative mode) gave 479.0884, theoretical ion distribution of which was $C_{22}H_{15}N_4O_9$. The results of FAB mass are consistent with the formulae (ketimine, 1, 2) in Chart 3. However, it cannot be determined from the present results whether the product is the ketimine or the quinonoid or a mixture of the two, since they have many sites of protonation or deprotonation in the molecules and the subtraction of only one proton at the methylene carbon of the PM moiety in the ketimine gives the quinonoid.

Conclusion

A quinonoid species absorbing in the 500-nm region was observed in the reactions of PM with PQQ, 1,7-PQ and 4,7-PQ in alkaline methanol. This may be the first example of metastable quinonoid species in pyridoxal catalysis which are not in the form of metal chelate. 1,10-PQ which lacks pyridine nitrogen peri to the *o*-quinone carbonyl groups does not

react with primary amines under the conditions used.

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