Resonance Raman Spectroscopy of Pyridoxal Schiff Bases*

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Resonance Raman (RR) spectra are reported for amino acid and amine adducts of pyridoxal 5'-phosphate (PLP) and 5'-deoxypyridoxal (5'-dPL) in aqueous solution. For the valine adducts, a detailed study has been carried out on solutions at pH 5 and pH 9, and 13, values at which the pyridine and imine protons are successively ionized, and on the adducts formed from 15N-valine, α-deuterovaline, and N-methyl-PLP. Good quality spectra were obtained, despite the strong fluorescence of pyridoxal Schiff bases, by adding KI as a quencher, and by exciting the molecules on the blue side of their absorption bands: 406.7 nm (cw Kr laser) for the pH 5 and 9 species (ν\text{max} = 409 and 414 nm), and 354.7 nm (pulsed YAG laser, third harmonic) for the pH 13 species (ν\text{max} = 360 nm). A prominent band at 1646 cm\(^{-1}\) is assigned to the imine C=N stretch via its 13 cm\(^{-1}\) \(^{15}\)N shift. A 12 cm\(^{-1}\) downshift of the band in D\(_2\)O confirms that the Schiff base linkage is protonated at pH 9. Deprotonation at pH 13 shifts \(\nu_{\text{C=N}}\) from 1646 to 1629 cm\(^{-1}\), values typical of conjugated Schiff bases. The strongest band in the spectrum, at 1338 cm\(^{-1}\), shifts to 1347 cm\(^{-1}\) upon pyridine protonation at pH 5, and is assigned to a ring mode with large component of phenol (5'-O) stretch. A shoulder on its low-frequency side is assigned to the C4-C4' stretch. Large enhancements of these modes can be understood qualitatively in terms of the dominant resonance structures contributing to the ground and resonant excited states. A number of weaker bands are observed, and assigned to pyridine ring modes. These modes gain significantly in intensity, while the exocyclic modes diminish, when the spectra are excited at 266 nm (YAG laser, fourth harmonic) in resonance with ring-localized electronic transitions.

Pyridoxal 5'-phosphate (PLP\(^{1}\)) is the essential cofactor of a large number of enzymes important in amine and amino acid metabolism (1–3). The mechanism and stereochemistry of PLP-dependent enzymatic reactions have been worked out in considerable detail, and a number of intermediates have been identified (1–3). In these enzymes, PLP is bound to a lysine sidechain via a Schiff base linkage (see Fig. 1 for the structure). During turnover, this linkage is transferred to the amine group of a substrate, and is the vehicle for subsequent chemical modification of the substrate.

Because of their extended conjugation, PLP Schiff bases absorb intensely in the near-UV region of the spectrum. This absorption band has been useful in monitoring the course of PLP reactions. It also offers the potential of probing the structure of PLP Schiff bases in situ via resonance Raman (RR) spectroscopy. This technique involves laser excitation at wavelengths close to allowed electronic transitions, producing strong Raman enhancement of vibrational modes which lead to the molecular distortion in the excited state (4). The chromophore vibrational spectrum can be recorded in dilute solution, unobscured by the vibrational spectrum of the surrounding protein (5, 6). This study of nonenzymatically formed Schiff bases of PLP provides essential background information for interpretation of the RR spectra of enzymes.

The strong fluorescence of PLP Schiff bases is a major obstacle, however, since it readily obscures the weaker Raman scattering. Ledbetter (7) has reported a few RR bands of PLP-valine Schiff base, seen on a sloping background. In the present study, good quality RR spectra of the PLP-valine Schiff base and several analogs have been obtained using potassium iodide as a fluorescence quencher (8). The vibrational spectra have been analyzed and partially assigned with the aid of isotopic frequency shifts for \(^{15}\)N and deuterium substitutions. The effect of deuteration at the C4' and C5' positions was determined via synthesis of labeled 5'-deoxy-pyridoxal. In the following study (9), these assignments are used to interpret resonance Raman spectra of a well-studied PLP-dependent enzyme, aspartate aminotransferase, and a novel photo-induced deuterium exchange at the C4' position of the PLP coenzyme is reported.

EXPERIMENTAL PROCEDURES

Compound Preparation

A number of previous syntheses have been published (10–12). We have based our procedures on Ref. 13. The synthetic route for preparing 5'-deoxypyridoxal, selectively deuterated at the 4' and 5' positions is given in Scheme 1.

4'-Deoxypyridoxine Hydrochloride (2) and 5'-Deoxypyridoxine Hydrochloride (3)

Pyridoxine hydrochloride (I; 5.00 g, 24.3 mmol) and 5% palladium on carbon (0.3 g) were mixed in 75 ml 0.3 N HCl. The mixture was hydrogenated at 40 p.s.i. for 17 h, then it was filtered through celite; the solvent was evaporated. The residue was dissolved in 200 ml of boiling absolute ethanol. Upon cooling to room temperature, compound 2 separated as colorless needles (1.35 g, 29% yield) melting at 260, 264, 265 °C (literature 13 m.p. 264–265 °C dec; 1H NMR (D-O) 5.242 (s, 3H, C-4'-H\(_3\)), 2.65 (s, 3H, C-2'-H\(_3\)), 4.81 (s, 2H, C-5'-H\(_2\)), 8.14 (s, 1H, C–6-H). A second crop of 2 (0.56 g, m.p. 259–261 °C dec) was obtained by cooling the mother liquor to -20 °C. The mother liquor was concentrated to dryness, and the residue was dissolved in 25 ml of boiling ethanol. A solution of 75 ml of ether gave white crystals of compound 3 (1.77 g, 38% yield) melting at 147–149 °C (literature

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1 The abbreviations used are: PLP, pyridoxal 5'-phosphate; RR, resonance Raman; 5'-dPL, 5'-deoxypyridoxal.
5'-Deoxyxypyrindine (4)

5'-Deoxypyridoxine hydrochloride (5; 379 mg; 2.00 mmol) was dissolved in 10 ml of water and heated to 70 °C. Sulfuric acid (0.1 ml) and manganese dioxide (180 mg; 2 mmol) were added, and the mixture was stirred at 70 °C for 2 h. The mixture was filtered through paper, and some acid was added. The resulting solution was extracted continuously with dichloromethane for 3 h. The organic extract was concentrated to dryness, and the oily residue was extracted with 10 ml of boiling hexane. Slow cooling of the hexane solution to -20 °C gave yellow needles of pure 5'-deoxypyridoxal (35 mg; 1.20 mmol) melting at 108-109.3 °C (literature (13) 108-109 °C; 'H NMR (DzO) δ 2.39 (s,3H, C-5'-H3), 7.97 (s, 1H, C-6-H). Sublimation of a portion of this material gave crystals melting at 107.5-109 °C.

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(13) 142-143 °C; 'H NMR (D2O) δ 2.39 (s,3H, C-5'-H3), 261 (s, 3H, C-2'-H3), 5.00 (s, 2H, C-4'-H2), 7.98 (s, 1H, C-6-H).

5'-Deoxyxypyrindine hydrochloride (6) and 5'-Deoxy[5'-2H]pyridoxine hydrochloride (6)

Pyridoxine hydrochloride (2.05 g; 10 mmol) and 5% palladium on carbon (0.2 g) were mixed in 15 ml of 0.6 N DCl in D2O and hydrogenated with deuterium gas at 40 psig for 24 h. Fractionation of the reaction mixture as described above gave compounds 5 (581 mg; 31% yield; m.p. 264-265 °C dec; 'H NMR (D2O) δ 2.41 (t, 2H, JH-H = 2, C-4'-H2), 4.81 (s, 2H, C-5'-H2), 8.14 (s, 1H, C-6-H) and 5 (845 mg; 41% yield; m.p. 147-149 °C; 'H NMR (D2O) δ 2.38 (t, 2H, JH-H = 2, C-5'-H2), 2.61 (s, 3H, C-2'-H3), 5.00 (s, 2H, C-4'-H2), 7.98 (s, 1H, C-6-H).

5'-Deoxy[5'-3H]pyridoxine hydrochloride (7)

5'-Deoxy[5'-2H]pyridoxine hydrochloride (6; 474 mg; 2.5 mmol) was oxidized with manganese dioxide as described above to give compound 7 (42 mg; 11% yield) melting at 107.5-109 °C; 'H NMR (CDCl3) δ 2.50 (3H, C-2'-H3), 2.56 (t, 2H, JH-H = 2, C-5'-H2), 7.97 (s, 1H, C-6-H); 10.39 (s, 1H, C-4'-H, 11.37 (br s, 1H, 3-OH); MS m/z 152 (M+, 100%), 151 (18), 122 (38), 95 (15), 83 (21). The NMR integration indicated that 87% of the molecules contained one deuterium at the C-5' position, and isotopic ratio analysis at the molecular ion in the mass spectrum yielded a value of 85%. The product showed a single component upon TLC analysis (solvent, 19:1, CHCl3:MeOH, Rf = 0.63. Sublimation of a portion of this material gave crystals melting at 108.5-108 °C.

Schiff Base—These solutions were prepared by dissolving pyridoxal 5'-phosphate (Sigma) or 5'-deoxypyridoxin in 0.1 M potassium phosphate H2O or D2O buffer to a concentration of 2 nM, adjusting the pH (pD) to 5, 9, or 13 with HCl (DCl) or NaOH (NaOD) and adding amino acid (glycine, DL-valine, DL-alanine, sodium glutamate) or amine (iso- and butylamine) to a final concentration of 0.2 mM (pH 5) or 0.5 mM (pH 5, 14, 15, 17). Because the Schiff base equilibrium is less favorable at pH 5 (14), only valine, isobutylamine, and N-butyamine, which have large formation constants (14), were used at this pH. The valine Schiff base was also prepared from N-methyl-PLP, which was kindly provided by Dr. Victor Chen, at Iowa State University. Isotopic derivatives were prepared with [15N]DL-valine (98.6% enrichment, Prochem) and DL-valine-2d5, (deuterovaline) (98% enrichment, Merck). For visible excitation Raman studies 3-4 M KI was added to the Schiff base solutions as a fluorescence quencher.

RESULTS AND DISCUSSION

Schiff Base Protonation States and Spectra

Fig. 1 is a structural diagram for a PLP Schiff base in acid solution. The pKb values for the three ionizable protons (14, 15, 17) are shown. UV visible absorption spectra are given for these three ionization states in Fig. 2, and are compared with
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Fig. 2. UV-visible absorption spectra of (a) pyridoxamine; (b) PLP-valine Schiff base at various pH. Concentrations = 0.5 mM, path length = 1 mm.

The spectrum of pyridoxamine. Fig. 3 shows RR spectra for the PLP-valine Schiff base at pH 9, where only the imine N is protonated. Valine was chosen for detailed study because it forms a particularly stable Schiff base adduct with PLP (14).

At pH 9, PLP-valine has a long wavelength absorption maximum at 414 nm, and fluoresces strongly with a maximum at 500 nm (18-20). The spectra in Fig. 3 were obtained using 406.7 nm excitation, with KI added to quench fluorescence. Residual fluorescence could still be seen, as a sloping base-line, and this was computer subtracted, as described under "Experimental Procedures." Also shown in Fig. 3 are spectra of the Schiff base in D_{2}O at pD 9, where the iminium proton is exchanged for a deuteron, and of the [^{14}N]valine and [^{2}H]valine adducts. PLP adducts of glycine, alanine, glutamate, and n-butylamine, prepared at pH 9, gave nearly identical spectra, except that the weak 1138-cm^{-1} band could be seen only for alanine. In Fig. 4, similar spectra are shown for 5'-deoxypyridoxal (5'-dPL)-valine Schiff base, selectively deuterated at the C4' and C5' positions.

Fig. 5 shows PLP-valine RR spectra at pH (pH) 5. At this pH the absorption and fluorescence bands are slightly blueshifted (\lambda_{max} = 409 and 495 nm) but good quality RR spectra could still be obtained via 406.7 nm excitation and KI quenching. All three ionizable sites are protonated at pH 5, and H/D exchange occurs at all three in D_{2}O. Phosphate protonation, however, is not expected to influence the resonance-enhanced Raman modes of the chromophore, and the spectrum of the N-methyl-PLP-valine Schiff base, in which pyridine protonation is blocked, showed no change between pH 9 (shown in Fig. 5) and pH 5. Pyridine protonation does affect the spectrum, however, as discussed below. Fig. 6 shows pH 5 spectra for 5'-dPL-valine.

Ultraviolet RR spectra of PLP-valine at pH 13 are shown in Fig. 7. At this basicity the iminium proton is neutralized, and the chromophore absorption and fluorescence maxima shift to 360 and 430 nm (18-20). The RR spectra were obtained with 354.7 and 266.0 nm excitation, using a pulsed YAG laser. For the 354.7 nm spectrum, KI was added to quench fluorescence, which was, however, insignificant at 266 nm. Different relative intensities for the RR bands are seen at the two wavelengths, reflecting different resonance enhancement mechanisms, which are discussed below. The effect is seen even more dramatically for PLP-valine at pH 9 and 5, for which UV RR spectra are compared with 406.7 nm spectra in Figs. 8 and 9. The altered intensities help to ascertain the frequencies of overlapping RR bands.

Band Assignments

Table I lists RR band frequencies, deuteration shifts, and suggested assignments for valine Schiff bases of PLP and 5'-dPL at pH (pD) 9, 5, and 13. In Table II, corresponding
frequencies for other Schiff base adducts of PLP are given. The assignments and the frequency correlations are based on isotopic frequency shifts and intensity correspondences, and on vibrational frequencies of related molecules. In the ensuing discussion, the cited frequencies are those of PLP-valine. Corresponding frequencies for 5'-dPL-valine are 5-15 cm⁻¹ lower; this systematic downshift is ascribable to the greater electron donating propensity of methyl relative to hydroxymethylphosphate substituent, leading to a general lowering of the bond orders in the aromatic system.

C=N Stretch—The prominent band at the high frequency end of the spectrum, ~1646-1650 cm⁻¹ at pH 5 and 9, and 1629 cm⁻¹ at pH 13, is attributable with certainty to the imine C=N stretch, since it shifts down ~11-18 cm⁻¹ when ¹⁵N-valine is incorporated. The frequencies correspond well with those observed for other protonated and neutral Schiff bases, including rhodopsin (21). As expected, this frequency is sensitive to deuteration at either end of the C=N bond. It shifts down by 8-12 cm⁻¹ upon N deuteration (as also noted by Ledbetter (7), and 16-19 cm⁻¹ upon C4' deuteration. These shifts are essentially additive: a 28-29 cm⁻¹ downshift is seen when both C and N protons are replaced by deuterium. This observation is important for the identification of photoinduced deuterium exchange at the C4' position of the PLP coenzyme in aspartate aminotransferase (9). The downshift in D₂O, due to H/D exchanged the imine N atom, confirms the inference from absorption and fluorescence (18-20) spectra that the tautomer stable in aqueous solution has the phenolic proton transferred to the imine N (see Fig. 10).

C-O Stretch—The strongest band in the PLP-valine RR spectrum is at 1338 cm⁻¹, shifting up 9 cm⁻¹ upon pyridine protonation (pH 5). We assign this band to a ring mode with a large component of phenolate C-O stretching. This mode is found at 1256 cm⁻¹ in phenol (22), and the much higher
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The frequency observed for PLP-valine is attributable to significant C=O double bond character via resonance form II, illustrated in Fig. 10. The upshift upon pyridine protonation suggests a stiffening of the C-O bond, via polarization. In the pH 13 spectrum, the prominent band in the midfrequency region is at 1304 cm⁻¹, and we assign this to the same C-O stretching mode. The substantial lowering of the frequency is consistent with the expected destabilization of resonance form II upon iminium deprotonation, which would place a negative charge at the imine N atom in form II. For 5'-dPL-valine the C-O stretch is 13 cm⁻¹ lower, suggesting a somewhat lower C=O contribution, due to π electron donation from the methyl substituent.

C4-C4' Stretch—The apparent frequency of the C-O stretching band is influenced somewhat by an overlapping band on its low frequency side. A clearly defined doublet is seen for several of the 5'-dPL-valine spectra (Figs. 4 and 6), whereas in other cases the low-frequency component is seen only as a shoulder, and sometimes cannot be seen at all. This variability appears to be due to a combination of frequency and intensity changes. We assign this low-frequency component to a mode with a large contribution from C4-C4' (pyridine-imine) stretch. The analogous benzene-vinyl stretch in styrene is observed at 1203 cm⁻¹ and shifts up, to 1225 cm⁻¹, upon deuteration of the vinyl (23). The higher frequency of the pyridine-imine stretch is again attributable to enhanced double bond character, associated with the resonance form II contribution. The band does shift to higher frequency upon C4' deuteration, as can be seen in the 5'-dPL-valine spectra at pH 5, and also pH 9; at pH 9 this upshift results in coalescence with the C-O stretch into a single broad band at an intermediate frequency.

Ring Modes—The remaining moderate to weak bands in the spectra are assignable to modes of the pyridine ring, identified via the benzene mode numbering system (see Table I). Not all of them are seen in every spectrum, but the various derivatives, taken together, produce a consistent pattern, as summarized in Table I. The bands at ~1590 and ~1540 cm⁻¹ at pH 9 are identifiable with ring modes $v_{a}$ and $v_{b}$, (derived from the degenerate benzene mode, $v_{c}$, which occurs at 1596 cm⁻¹). $v_{a}$, but not $v_{b}$, must also have a substantial imine component, since it shows a large (~20 cm⁻¹) downshift upon imine deuteration (pD 9). Both bands show large (30-50 cm⁻¹) upshifts upon pyridine protonation as do the corresponding modes of pyridine itself (24). The pair of bands at 1473 and 1465 cm⁻¹ (1467, 1450 cm⁻¹ for 5'-dPL) are assigned to ring modes $v_{14'}$ and $v_{15'}$ (24). $v_{14'}$, but not $v_{15'}$, shows a large (~40 cm⁻¹) downshift upon imine deuteration, again reflecting a
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PLP-VAL pH 9

Fig. 8. Resonance Raman spectra of the pyridoxal phosphate-valine Schiff base complex at pH 9. a, 406.7 nm excitation; b, 354.7 nm excitation; c, 266.0 nm excitation.

The very weak band at 1256 cm⁻¹ is tentatively assigned to ring mode ν3. A prominent band is seen at 1195 cm⁻¹ in pH 9 solutions, and is assigned to ring mode ν19. This band shifts up significantly, to 1202 cm⁻¹, upon pyridine protonation, and shifts up further, to ~1214 cm⁻¹, upon pyridinium H/D exchange. This behavior is likewise seen for a band in the 1200 cm⁻¹ region of uracil, and other nitrogen heterocycles, and is attributable to a change in the normal mode composition, involving contributions from C-N stretching and N-H(D) bending (26).

A pair of weak bands at 1138 and 1103 cm⁻¹, are assigned to ring modes ν15 and ν18b. The latter shifts down significantly upon imine deuteration, but up on C₄′ deuteration, and apparently involves a complex admixture of imine coordinates. The 1138 cm⁻¹ band may also contain a contribution from stretching of the bond from the imine N atom to the valine Cα atom, since it appears to shift up to 1150 cm⁻¹ in the spectrum of the PLP-α-D-valine derivative. There does not, however, appear to be any significant shift in the PLP-[¹⁵N]valine spectrum, so this assignment is uncertain.

Electronic Spectra and Resonance Enhancement

The absorption spectrum of pyridoxamine (Fig. 2) is closely related to that of benzene. The weak bands at 313 and 244 nm correspond to the first and second singlet excitations (B₁u, B₂u) of benzene, which are symmetry forbidden, while the strong band at ~200 nm corresponds to the third benzene singlet (E₁g), which is allowed. These same bands are seen, somewhat red-shifted, for the PLP-valine Schiff base, but in addition there is a strong long wavelength absorption, at 402, 410, and 360 nm at pH 5, 9, and 13, associated with the extended pyridine-imine conjugation. The ground and first
the spectra. Its weakness in others may be due to intensity also be enhanced, and this band is indeed strong in some of from a transition between forms excited state of this chromophore contain contributions from resonance states of the PLP-valine RR spectra when excited in the longest wavelength absorption band; the ring modes are all substantially weaker.

The situation is very different at 266 nm, in resonance with the ring-localized excitations, where the ring modes are seen to gain substantially in relative intensity (Figs. 7-9). The longest wavelength absorption band; the ring modes are all substantially weaker.

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provide for enhancement of two-quantum vibrational transitions, leading to the observation of overtones and combinations of $v_R$ and $v_O$ (29). This mechanism might account for the intensification in our 266-nm PLP-valine RR spectra of a band at 1402–1416 cm$^{-1}$ which we have tentatively assigned to $2v_C$.

In relation to the ring modes, the exocyclic stretching modes decrease in intensity at 266 nm. This is particularly striking for the C–O stretch, which is the strongest band in the long wavelength spectra, but becomes quite weak at 266 nm, especially for the pH 9 sample (Fig. 8).

Summary

When excited in their long wavelength absorption bands, pyridoxal Schiff bases display strong enhancement of modes assignable to stretching of the exocyclic pyridoxime bonds, C=N, C-C, and C-O, consistent with the expected bond length changes in the first excited state, formed by the extended conjugated system. The frequencies of these bands are sensitive to the protonation state of both the imine and pyridine N atoms, and are useful markers for these structural features. It can be anticipated that in pyridoxal enzymes these frequencies can be used to monitor interactions of the pyridoxal coenzyme with its protein environments, which may influence the protonation state or otherwise perturb the conjugated system. The imine stretch is sensitive to deuteration at both the N and C ends of the C=N bond, and can be used to detect H/D exchange at these positions in pyridoxal enzymes.

A number of weaker RR bands are seen, which can be satisfactorily assigned to various pyridine ring modes. With 266-nm excitation, in resonance with electronic transitions that are expected to be localized on the pyridine ring, the ring modes are significantly enhanced, whereas the exocyclic modes diminish in intensity. Particularly strong enhancement is seen for a pair of ring modes in the 1590 cm$^{-1}$ region, which are assigned to ring modes $v_R$ and $v_O$. Their prominence can be related to the strong vibronic activity of the parent mode $v_R$ benzene, which is strongly enhanced upon excitation in the second singlet band of substituted benzenes.

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REFERENCES

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