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Spectroscopic Studies of Quinonoid Species from Pyridoxal 5′-Phosphate†

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ABSTRACT: To establish the state of protonation of quinonoid species formed nonenzymically from pyridoxal phosphate (PLP) and diethyl aminomalonate, we have studied absorption spectra of the rapidly established steady-state mixture of species. We have evaluated the formation constant and the spectrum of the mixture of Schiff base and quinonoid species. For *N*-methyl-PLP a singly protonated species with a peak at 464 nm is formed from the unprotonated aldehyde and the conjugate acid of diethyl aminomalonate with a formation constant K_f of 240 M^{-1}. The very intense absorption band with characteristic vibrational structure (most evident as a shoulder at 435 nm) is accompanied by a weaker, structured band at about 380 nm and a weak, broad band at 330 nm. We suggest that the 380-nm band may represent a tautomeric form of the quinonoid compound. Protonation of the phosphate group appears to affect the spectrum only slightly. The corresponding mixture of Schiff base and quinonoid species formed from PLP has a very similar spectrum at pH 6–7. It has a formation constant K_f of 230 M^{-1} and a pK_a of 7.8, which must be attributed to the ring nitrogen atom. The dissociated species, which may be largely carbanionic, has a strong structured absorption band at 430 nm and a weaker one, again possibly a tautomer, in the 330-nm region. The analysis establishes that in all species a proton remains on either the phenolic oxygen or the imine nitrogen. Proton NMR spectroscopy, under some conditions, reveals only two components: free PLP and what appears to be Schiff base. However, we suggest that the latter may, in fact, be a quinonoid form, either alone or in rapid equilibrium with the Schiff base. Absorption spectra of quinonoid species formed in enzymes are analyzed and compared with the spectra of the nonenzymic species.

A generally accepted mechanism of catalysis by pyridoxal 5′-phosphate (PLP)[1] dependent enzymes postulates formation of a Schiff base (II; Scheme I) between the PLP (I) and an amino acid substrate. This is followed by withdrawal of an electron pair from a suitably oriented bond around the α carbon atom into the conjugated system represented by the imine double bond and the pyridine ring (Braunstein &

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SB, Schiff base; Q, quinonoid species.

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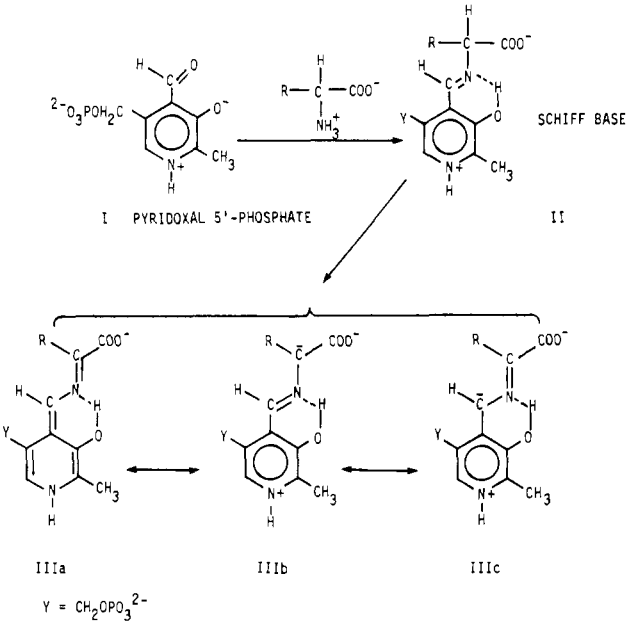
Scheme I



Shemyakin, 1953; Metzler et al., 1954a). The electron-accepting properties of the pyridine ring are apparently essential and, furthermore, can be enhanced by protonation of the ring nitrogen.

If the ring nitrogen is protonated, cleavage of the α C–H bond leads directly to the quinonoid structure IIIa (Scheme I; Metzler et al., 1954a). The two dipolar ionic forms IIIb and IIIc can be regarded as resonance forms of IIIa. If the ring nitrogen is unprotonated, the form corresponding to IIIa presumably contributes less while forms analogous to IIIb and IIIc contribute more to the structure of the resulting anion (Martell, 1982).

Jenkins reported in 1960 an intense light absorption band at 492 nm with pure cytosolic aspartate aminotransferase and an impurity present in glycine. The impurity turned out to be *erythro*-3-hydroxyaspartate (Jenkins, 1961). Jenkins suggested that the 480-nm band represented quinonoid IIIa. Later Schirch and Slotter (1966) observed a similar absorption band at 480 nm with a molar absorptivity of about 40 000 when they mixed *N*-methylpyridoxal with diethyl aminomalonate in ethanol. Reaction of diethyl aminomalonate with pyridoxal gave a red precipitate that Abbott and Bobrick (1973) and more recently Sala et al. (1987) have characterized by infrared spectroscopy as quinonoid IIIc. Studies of PLP + diethyl aminomalonate have been conducted by Thanassi and Fruton (1962) and by Thanassi (1975). After a slow hydrolysis of the diethyl aminomalonate to its half-ester, PLP catalyzes a rapid decarboxylation. However, when PLP is present initially, an intense quinonoid band develops and hydrolysis of the ester is retarded.

Matsumoto and Matsushima (1972, 1974) reported a series of studies of quinonoid species that appear in methanolic solution in the presence of Al(III) salts. Thus, ethyl pyruvate + pyridoxamine + aluminum nitrate in a 1:1:1 molar ratio gradually reacted to give a 488-nm band. The same species, together with Schiff base, could be formed from alanine ethyl ester + pyridoxal + Al^{3+}, but no corresponding bands are formed in aqueous solutions. Karube and Matsushima (1976) showed that tryptophan + *N*-methylpyridoxal + Al^{3+} in methanol gave a broad band at 467 nm, presumably an aluminum chelate of a Schiff base of 2-aminoacrylate, the product of β-elimination of indole. This gradually gave rise to a band at 514 nm thought to be quinonoid. Karube and Matsushima (1977) observed a quinonoid band at 550 nm arising from 2-aminobutenoic acid + *N*-methylpyridoxal + Al^{3+} in methanol.

A relatively small amount of NMR spectroscopy relevant to the quinonoid structures has been conducted. Abbott and Martell (1973) and Martell and Taylor (1984) reported that a solution of pyridoxal + alanine + Al^{3+} in a 1:1:1 ratio in D_2O at 10 °C, pD 5.0, has, in addition to the 4′-H resonance of the Schiff base at 9.0 ppm, a small resonance at about 8.8 ppm representing about 10% of the total pyridoxal. It forms slowly and decays over a period of hours. The authors suggested that it represents the Al(III) chelate of a carbanionic form, a resonance hybrid of IIIb and IIIc. However, Martin (1987) has shown that the kinetics of its appearance and decay are not those expected for an intermediate species. Sala et al. (1987) dissolved the precipitated quinonoid compound formed from pyridoxal and diethyl aminomalonate in dimethyl sulfoxide and observed NMR resonances appropriate for a Schiff base rather than the quinonoid.

The expected products of the decay of a quinonoid intermediate are amine or aldehyde, e.g., pyridoxamine 5′-phosphate (PMP) or PLP. In addition, the quinonoid intermediate can also readily undergo aldol condensation with aldehydes. Thus, pyridoxal and glycine can give β-pyridoxylserine (Metzler et al., 1954b), and PLP catalyzes condensation of either aminomalonate or monoethyl aminomalonate with formaldehyde, acetaldehyde, or benzaldehyde to give serine, threonine, or β-phenylserine, respectively (Matthew & Neuberger, 1963). Diethyl aminomalonate was found much less reactive. However, Thanassi (1975) observed condensation of diethyl aminomalonate with PLP itself to give β-phosphopyridoxylserine.

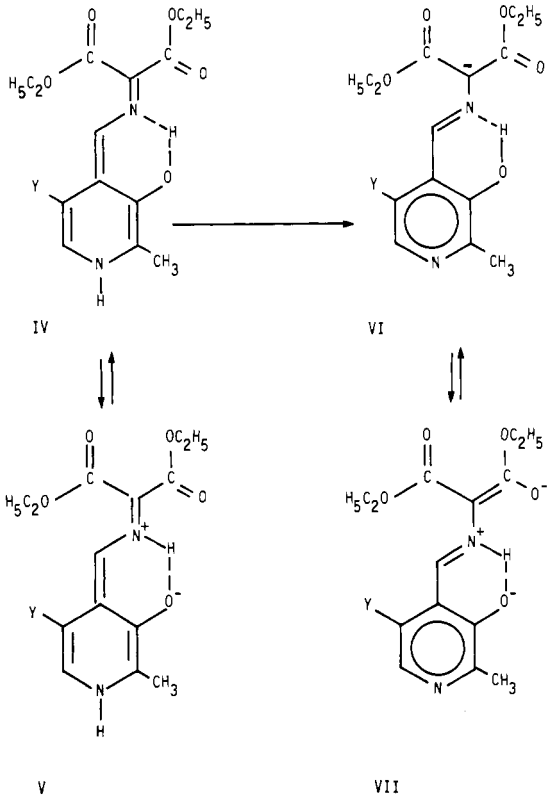
In addition to aspartate aminotransferase a number of other enzymes give well-defined quinonoid absorption bands, usually with amino acids that act as competitive inhibitors or as quasisubstrates. These include tryptophanase + alanine (500 nm; Morino & Snell, 1967), tryptophanase + ethionine (508 nm; June et al., 1981), tryptophan synthase + L-serine + *O*-methylhydroxylamine (466 nm; Dunn et al. 1987), serine hydroxymethyltransferase + glycine (Ulevitch & Kallen, 1977; Ching & Kallen, 1979), γ-cystathionase + homoserine lactone 498 nm), + homoserine thiolactone (525 nm), or + homoserine selenolactone (530 nm; Brown et al., 1969). Tryptophan synthase with its substrate L-serine and indole gives a quinonoid band at about 460 nm (Drewe & Dunn, 1986).

Despite the many studies, a number of questions have been raised about these quinonoid absorption bands. There is always a prominent shoulder on the high-energy (short-wavelength) side of the band and in some cases, e.g., tryptophan synthase and γ-cystathionase, a less noticeable shoulder on the low-energy side. Do these bands all belong to a single chemical species? Why do some quinonoid bands absorb at 500 nm or higher while others absorb at as low as 459 nm? Do these have the same state of protonation? How do tautomers IV and V (Scheme II) differ in spectral properties? In an attempt to answer some of these questions we have studied the rapidly established equilibria in mixtures of PLP and diethyl aminomalonate by electronic and proton NMR spectroscopy and have also compared spectra of various quinonoid species.

Materials and Methods

Materials. Pyridoxal 5′-phosphate (PLP) and diethyl aminomalonate were purchased from Sigma and were used without further purification. Pyridoxal 5′-phosphate *N*-methochloride and O^3-methyl-PLP were synthesized according

Scheme II



IV VI

V VII

to the method of Chen (1981) using the procedures of Pocker and Fischer (1970); 5-deoxypyridoxal was synthesized by the method of Iwata (1968).

Procedures. Absorption spectra were recorded on a Cary Model 1501 spectrophotometer with the On-Line Instruments modification that permits direct recording of spectra by a computer. Absorbances were normally recorded at intervals of 50 cm^{-1}, and the base line was subtracted automatically. After conversion to apparent molar absorptivity, spectra were transferred to the Iowa State University computation center for numerical analysis. pH measurements were made with a Radiometer PHM 84 pH meter. NMR spectra were recorded with a Bruker WM-300 spectrometer at 300 MHz (spectral width, 4000 Hz; acquisition time, 2 s; flip angle, 20°) and on a Bruker WM-200 at 200 MHz.

All samples were mixed and run as rapidly as possible. Stock solutions of 5×10^{-4} M PLP and 1 M diethyl aminomalonate were prepared immediately before use. The appropriate volume of diethyl aminomalonate was then mixed with water containing buffered ethylenediaminetetraacetic acid. Then NaOH or HCl was added in the appropriate quantity to give the desired final pH, and PLP or *N*-methyl-PLP was added last. The solution was brought to volume rapidly in a 5-mL volumetric flask, mixed, and transferred to a cuvette. After the final addition, usually only 50–60 s elapsed before recording of a spectrum was begun. Spectra were recorded at three or more times, each scan taking about 3 min. The first two scans were used to extrapolate to zero time by using a program that computed a correct time for each spectral point and carried out a linear extrapolation. Some spectra were also followed for longer times. Most of the experiments of Matsumoto and Matsushima (1972, 1974) with pyridoxamine + ethyl pyruvate and with pyridoxal + ethyl alaninate were repeated exactly as described. Hydrochlorides of pyridoxal and of ethyl alaninate were dissolved in methanol individually, and an equimolar amount of KOH was added to each. These solutions were then mixed together. Small amounts of methanolic KOH were added to give the spectra in Figure 7.

For NMR measurements the diethyl aminomalonate hydrochloride and PLP or *N*-methyl-PLP were separately lyophilized from 99.8% D_2O 3 times. They were redissolved in D_2O, and the pH of each solution was raised to about 6.2 with NaOD. The apparent pH (uncorrected pH meter reading) was adjusted after mixing and recording of the spectra was begun within 5–30 min.

Results

In Figure 1 the band shapes of a variety of quinonoid species are compared. These include bands derived from aspartate aminotransferase (A) + *erythro*-3-hydroxyaspartate, (B) with O^3-methyl-PLP in the active site + 2 M L-aspartate (Chen et al., 1987), (C) with 6-fluoro-PLP in the active site + *erythro*-3-hydroxyaspartate (Scott et al., 1986), and (D) *Escherichia coli* tryptophanase–L-alanine complex. The band parameters (peak position, height, width, and skewness) are given in Table I for the various lognormal bands fitted to these spectra. The similarities are obvious. The bands are unusually narrow for aromatic spectra (Davis & Metzler, 1972; Metzler & Metzler, 1987) with widths at half-height of $1.3–1.7 \times 10^3$ cm^{-1} and resembling spectra of dyes rather than of Schiff bases of PLP or of other typical benzenoid compounds. All can be represented as a series of two or three overlapping Gaussian or nearly Gaussian subbands spaced at intervals of about 1000–1100 cm^{-1}. However, there are differences, for example, in the relative heights of the main peak and of the major shoulder.

The clearest of the quinonoid bands are those of aspartate aminotransferase containing 3′-O-methylated PLP and L-aspartate and of tryptophanase + L-alanine. There are no other absorption bands on the immediate high-energy side of the band with *O*-methyl-PLP. The tryptophanase–alanine complex has only a weak Schiff base band in this region. Both quinonoid species have a shoulder 36–42% as high as the main band and a second shoulder 11–14% as high.

When either PLP or N-methylated PLP is mixed with an excess of diethyl aminomalonate, the quinonoid band, which peaks at 460 nm, appears within a few seconds and fades over a period of hours with appearance of an absorption band in the 330-nm region (Figure 2). Both the formation and disappearance of the 460-nm band are decreased by addition of ethylenediaminetetraacetic acid (EDTA). Since the decay seemed to be slowed more than the formation, we routinely added 5 mM EDTA to solutions. However, data obtained in its absence were qualitatively the same as those shown here.

Although the procedure is approximate, we have made, for each experimental solution, a linear extrapolation to zero time using pairs of spectra recorded a few minutes after mixing. At high concentrations of diethyl aminomalonate the peak absorbance is reached in less than 11 s. Therefore, these extrapolated data represent approximately the spectra of the products that are formed nearly instantaneously. As can be seen from the spectra of Figure 3, which are calculated from the extrapolated data, at low pH an intense band appears with a molar absorptivity of about 30 000–34 000 M^{-1} cm^{-1} at 459 nm. The shoulder at about 430 nm is relatively high, but the absorption then drops sharply. There is always a series of weak bands on the high-energy side. However, from their positions most of them appear not to be part of the same electronic transition as the intense band. It is to be expected that there will also be small amounts of free PLP (absorbing partly at 390 nm and partly at lower wavelengths) and of Schiff base (absorbing at about 420 nm).

Table I: Positions and Shapes of Various Quinonoid Absorption Bands of Pyridoxal Phosphate Resolved with Lognormal Distribution Curves

source	band maximum nm	band maximum $cm^{-1} \times 10^{-3}$	height (M^{-1} cm^{-1})	width ($cm^{-1} \times 10^{-3}$)	skewness	area ($m/mol \times 10^{-6}$)
AspAT + *erythro*-3-hydroxyaspartate	491	20.35	23.7	0.98	1.00	247
	464	21.40	10.2	1.07	1.00	116
	447	22.39	3.7	1.56	1.00	62
ApoAspAT						
+O^3-methyl-PLP	481	20.78	34.7	0.95	1.00	350
+2 M L-aspartate	459	21.80	12.5	1.06	1.00	140
	440	22.74	3.8	1.24	1.00	49
ApoAspAT						
+6-F-PLP	495	20.19	19.5	1.27	1.10	263
+*erythro*-3-hydroxyaspartate	469	21.30	11.1	1.29	1.10	153
	445	22.48	3.6	1.35	1.10	52
tryptophanase	501	19.96	21.98	0.86	1.10	201
+L-alanine	477	20.99	9.27	1.13	1.10	111
	453	22.08	2.98	1.64	1.10	52
N-CH_3-PLP + diethyl aminomalonate $(SB + Q)^{2-}$	464	21.55	32.8	1.46	1.00	510
	435	22.98	18.8	1.49	1.00	298
	410	24.41	6.7	1.52	1.00	109
	385	25.95	6.0	1.60	1.00	102
	365	27.42	4.8	1.60	1.00	82
	347	28.86	2.4	1.60	1.00	40
	327	30.60	2.3	3.53	1.35	89
PLP + diethyl aminomalonate $H(SB + Q)^{2-}$	459	21.77	22.6	1.46	1.00	351
	430	23.23	12.1	1.49	1.00	192
	405	24.69	3.9	1.52	1.00	63
	380	26.25	4.1	1.60	1.00	70
	361	27.69	3.6	1.60	1.00	61
	344	29.06	1.9	1.60	1.00	33
	327	30.60	1.9	3.53	1.35	74
PLP + diethyl aminomalonate $(SB + Q)^{3-}$	442	22.58	12.2	1.61	1.00	209
	417	23.99	10.8	1.65	1.00	189
	394	25.40	7.6	1.69	1.00	137
	367	27.24	6.1	1.90	1.00	124
	346	28.93	4.6	1.94	1.00	96
	326	30.67	3.2	2.04	1.00	69
PLP + diethyl aminomalonate in 100% methanol	468	21.35	5.4	1.46	1.00	84
	441	22.68	3.2	1.60	1.00	54
	413	24.19	1.1	1.60	1.00	19
	387	25.81	1.0	1.70	1.00	18
	368	27.20	0.45	1.70	1.00	8
	350	28.60	0.17	1.70	1.00	3
	340	29.43	9.6	4.53	1.39	472
5-deoxypyridoxal + diethyl aminomalonate	457	21.90	19.4	1.46	1.00	302
	429	23.30	11.9	1.49	1.00	188
	406	24.66	4.6	1.52	1.00	75
	383	26.08	4.2	1.60	1.00	71
	362	27.59	3.6	1.60	1.00	62
	344	29.06	1.3	1.60	1.00	22
	327	30.60	2.8	3.53	1.35	109
pyridoxal + alanine ethyl ester + $Al(NO_3)_3$ in methanol in 1:1:1 ratio (1×10^{-4} M)	486	20.59	12.6	1.34	1.00	180
	455	21.98	4.9	1.34	1.00	70
	428	23.35	1.6	1.34	1.00	22
	402	24.89	0.5	2.15	1.40	12
	372	26.86	0.8	1.44	1.00	12
	355	28.19	1.0	1.44	1.00	15
	339	29.51	0.8	1.44	1.00	12
	311	32.15	1.3	3.57	1.40	51
	283	35.27	4.1	4.32	1.40	195
5-deoxypyridoxal + alanine ethyl ester + $Al(NO_3)_3$ in methanol	486	20.59	9.836	1.300	1.00	136
	456	21.91	4.232	1.33	1.00	60
	430	23.23	1.918	1.36	1.00	28
pyridoxal (8.9×10^{-5} M) + alanine ethyl ester (8.9×10^{-4} M) + $Al(NO_3)_3$ (2.2×10^{-4} M)[a]	496	20.16 ±0.01	1.00[b]	1.28 ±0.02	1.00	
	466	21.47	0.35[b] ±0.02	1.30	1.00	
	446	22.43 ±0.02	1.00[b]	1.27	1.00	
	423	23.65	0.50[b] ±0.04	1.30	1.00	
		25.23		3.36	1.41	
		29.78		4.86	1.45	

[a] The values given are averages for the two spectra of Figure 7 and an additional spectrum at an apparent pH of 6.7. [b] The relative heights of the two pairs of Gaussian bands are given.

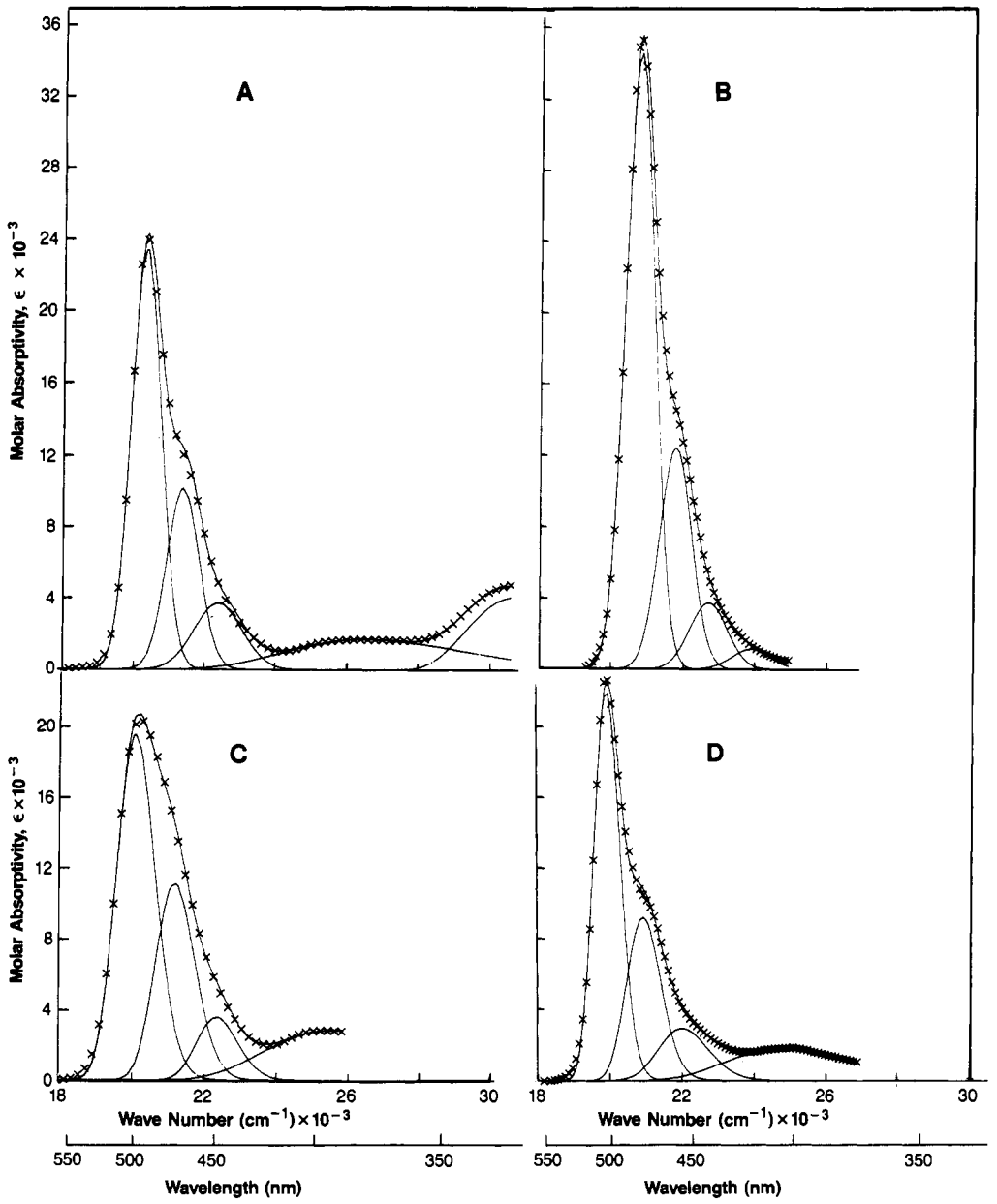


FIGURE 1: Spectra of various enzymic species thought to be quinonoid derivatives of pyridoxal phosphate (PLP): (A) Cytosolic aspartate aminotransferase of pig hearts with 20 mM *erythro*-3-hydroxyaspartate. (B) Cytosolic aspartate aminotransferase with the coenzyme replaced by O^3-methyl-PLP in the presence of 2 M L-aspartate [from Chen et al. (1987)]. (C) Cytosolic aspartate aminotransferase with the coenzyme replaced by 6-fluoro-PLP + 5 mM *erythro*-3-hydroxylaspartate. (D) Tryptophanase from *E. coli* in the presence of 0.2 M L-alanine (courtesy of Rukmani Viswanath). Each spectrum has been approximated as the sum of several Gaussian and lognormal curves. Three such bands, with shape parameters given in Table I, are used to approximate each quinonoid band. The broader bands represent other forms of the coenzyme in equilibrium with the quinonoid form in the active sites of the enzymes. The solid lines passing through or close to the experimental points represent the sums of the component bands.

To obtain the spectra of Figure 3A we used *N*-methyl-PLP, which gives the same spectrum with diethyl aminomalonate at any pH from about 5.1 to 9.3. From measurements with various concentrations of both the aldehyde and diethyl aminomalonate at several pH values, we used the extrapolated spectra to evaluate (Nagano & Metzler, 1967; Metzler et al., 1980) the equilibrium constants and the spectrum of the Schiff base–quinonoid mixture according to eq 1. Here all reactions

$$\begin{array}{ccccc} H_2P & \xrightarrow{3.68} & HP^- & \xrightarrow{6.1} & P^{2-} \\ & & + & & \\ & & AmH^+ & \xrightarrow{5.4} & Am \\ & & & & \downarrow \log K_f = 2.4 \\ H(SB + Q)^- & & \xrightarrow{6.1} & & (SB + Q)^{2-} \end{array} \qquad (1)$$

are reversible, and the arrows indicate the directions for which the equilibrium constants are defined. The numbers are pK_a values. (P = unprotonated *N*-methyl-PLP; Am = diethyl aminomalonate; SB + Q = sum of Schiff base and quinonoid forms.)

The low pK_a value of 3.64 for *N*-methyl-PLP was evaluated spectrophotometrically and agrees with values of 3.68 (Chen, 1981) and 3.70 (Pocker & Fischer, 1969). The pK_a of the phosphate group was set at 6.1 for evaluation of the spectra of the three ionic forms P, HP, and H_2P. The apparent pK_a of diethyl aminomalonate was determined by electrometric titration of a 1 M solution of the hydrochloride with NaOH. As we anticipated, the dissociation of the phosphate group, at a pK_a assumed to remain at 6.1, had only a small effect on the spectrum of the SB + Q mixture, which is shown in Figure 3. In setting up the equations for computer-assisted analysis, it is important to choose a pH-independent equilibrium be-

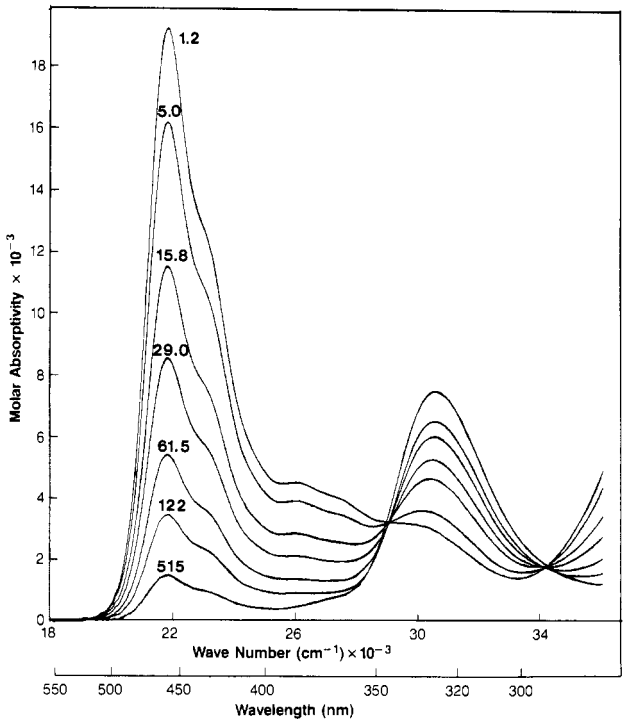
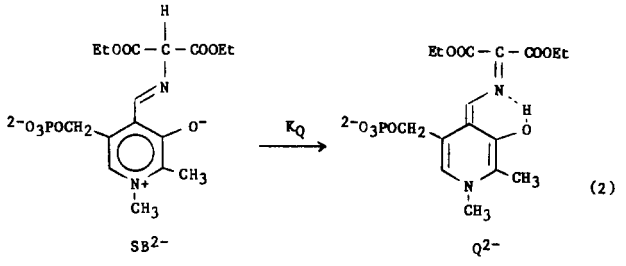


FIGURE 2: Spectrum of a mixture of 4.96×10^{-5} M PLP + 0.02 M diethyl aminomalonate at pH 6.2 versus time. The time in minutes is given by each curve.

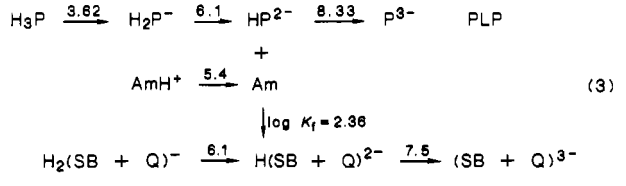
tween free aldehyde, amine, and Schiff base for evaluation of the formation constant K_f (eq 1). This condition also requires that the equilibrium between SB^{2-} and Q^{2-} be independent of pH. Thus, the proton removed from the α carbon of the Schiff base appears in the phenolic group of the quinonoid form (eq 2). The tautomerization constant K_Q is the ratio $[Q^{2-}]/[SB^{2-}]$.



(2)

The spectra of these two species in the proportions given by K_Q will determine the overall spectrum labeled $(SB + Q)^{2-}$ in Figure 3.

The PLP–diethyl aminomalonate system is more complex because the N-protonated Schiff base can dissociate. Additional components must be added to the scheme in eq 1 as follows (eq 3).



(3)

Here $H(SB + Q)^{2-}$ corresponds to the similarly charged N-methylated form of eq 1. The extrapolated zero-time spectra for the PLP–diethyl aminomalonate system for several experimental solutions varied with pH in a clear manner with an approximate isosbestic point at about 442 nm (Figure 4). Because of the previously discussed experimental difficulties, this isobestic point is not sharp. Nevertheless, the system can be described well by eq 3.

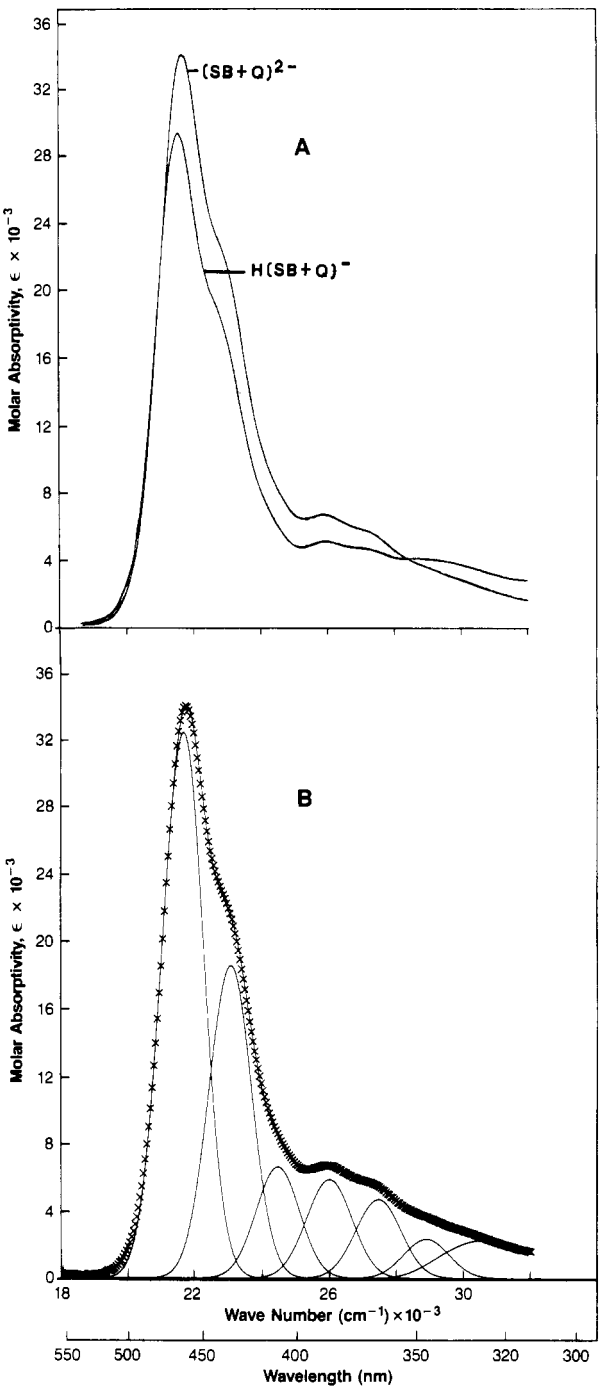


FIGURE 3: Calculated spectra for ionic forms of mixed Schiff base + quinonoid species from diethyl aminomalonate + *N*-methyl-PLP. (A) Spectra as evaluated by extrapolation to zero time and with log K_f (eq 1) = 2.4; $H(SB + Q)^-$, with protonated phosphate group; $(SB + Q)^{2-}$, unprotonated form. (B) Form $(SB + Q)^{2-}$ resolved with lognormal distribution curves.

The diethyl aminomalonate was always in large excess over PLP. Therefore, if all products formed contained a 1:1 ratio of PLP to diethyl aminomalonate, there should be no concentration dependence of the equilibrium spectra when expressed as molar absorptivity. In fact, this was found true for the concentrations used in the spectrophotometric studies. Figure 5 shows the spectra of the three forms $H_2(SB + Q)^-$, $H(SB + Q)^{2-}$, and $(SB + Q)^{3-}$ of the PLP–diethyl aminomalonate system. The computed spectra for 1.14×10^{-4}, 0.448 $\times 10^{-4}$, and 4.48×10^{-6} M (recorded in 10-cm cells) PLP are nearly identical when plotted as molar absorptivities. We conclude that no significant amount of any aldol condensation

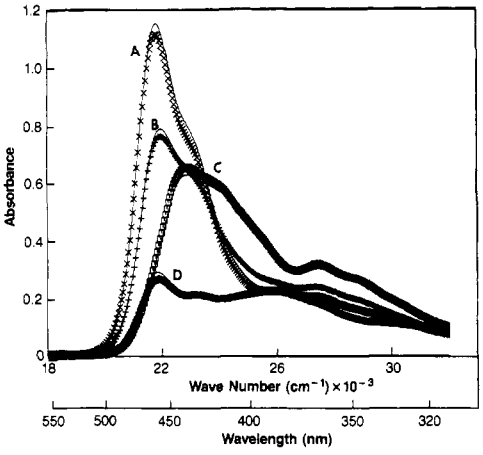


FIGURE 4: Representative comparison plots for data to obtain the calculated spectra of Figure 5 and the equilibrium constants for eq 3. Each experimental curve (data points) is compared with a theoretical curve (solid lines). Plots shown are for (a) pH 6.02, (b) pH 7.59, and (c) pH 8.95, all at 0.2 M diethyl aminomalonate, and (d) pH 6.72 at 0.002 M diethyl aminomalonate.

products or other 2:1 species absorbing in the 330-nm range is formed. Therefore, we believe that Figures 3 and 5 represent accurately the mixture of quinonoid plus Schiff base species present.

When incorporated into the active site of aspartate aminotransferase, *O*-methyl-PLP gives an intense quinonoid band with aspartate (Figure 1). However, with diethyl aminomalonate in water it gave only a very weak, rather broad band at 410 nm. Possibly as a result of the inability to form a strong hydrogen bond between the 3′-*O* and the imine nitrogen, no quinonoid species comparable to that obtained with PLP is formed.

The 327-nm band, which is formed when solutions of PLP or *N*-methyl-PLP and diethyl aminomalonate are allowed to stand and the quinonoid band disappears (Figure 2), presumably includes pyridoxamine 5′-phosphate (PMP) or *N*-methyl-PMP. It can also include products of aldol condensation and others. We can estimate the "molar areas" (integrated intensities) of the decaying quinonoid bands from the decrease in area relative to the increase at 327 nm. This was done by resolving the curves with Gaussian and lognormal distribution curves as in Figures 3B and 6. Between 1.25 and 122 min, sharp isosbestic points are maintained at 344 and 293 nm (Figure 2). We measured the changes in band areas from the analysis shown in Figure 6. The total area of the quinonoid bands (six Gaussian components) fell from 672 × 10^6 m/mol (672 Mm/mol) to 59 Mm/mol during this time while the area of the 327-nm band rose from 103 to 281 Mm/mol. The ratio of quinonoid area lost to the gain at 327 nm is 3.44. We estimate that if the quinonoid band had decayed completely, the final area of the 327-nm band would have been increased to 295 Mm/mol. This is acceptably close to the 346 Mm/mol observed for PMP. The position and shape of this band also match those of PMP closely. We conclude that the molar area a° of the quinonoid bands (six Gaussian components) is 3.44 × 295 (or 346) = 1015 (1190) Mm/mol.

We have also compared the systems described in the preceding paragraphs with the pyridoxal–alanine ethyl ester–$Al(NO_3)_3$ system of Matsumoto and Matsushima (1972, 1974). We repeated their basic experiments and obtained exactly the results described in the literature. The quinonoid band generated from pyridoxal (or 5-deoxypyridoxal) has a shape and position similar to that seen with enzymes (Table I). Matsumoto and Matsushima (1974) reported that when a 10:1 ratio of alanine ethyl ester to pyridoxal was used, the spectral peak was shifted 12 nm to a longer wavelength than for a 1:1 ratio. When KOH was added in small amounts, the rapidly fading bands were shifted to a shorter wavelength. As the apparent pH was raised, the peak shifted from 496 to 449 nm. We analyzed spectra obtained in this manner for apparent pH values of 6.3, 6.7, and 7.0 using pairs of lognormal curves. Two of these are shown in Figure 7, and the average band parameters are included in Table I.

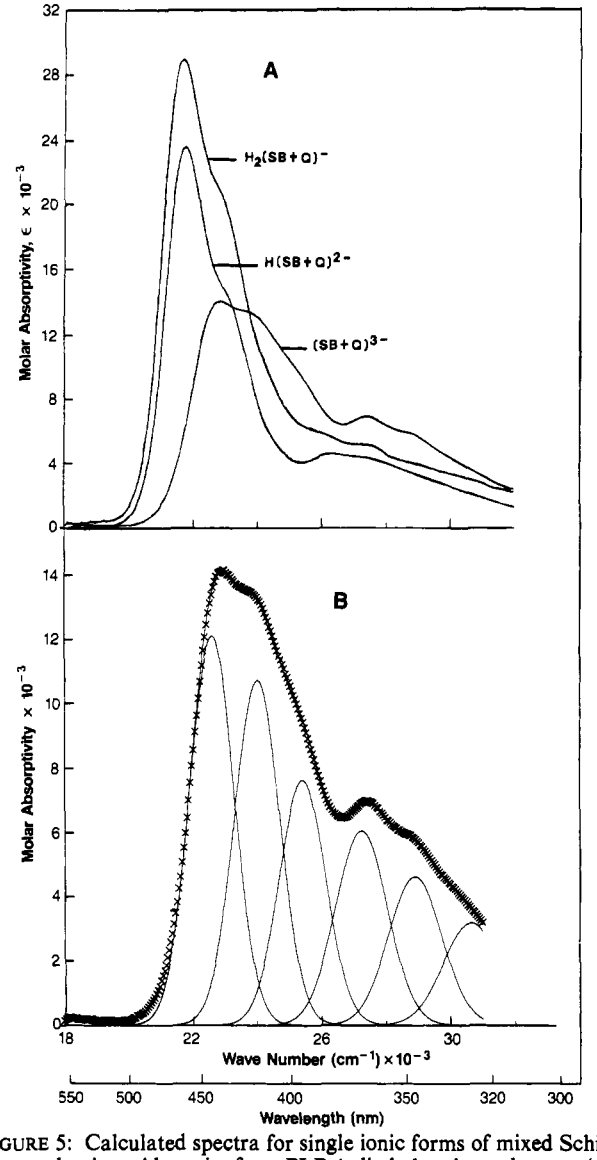


FIGURE 5: Calculated spectra for single ionic forms of mixed Schiff base and quinonoid species from PLP + diethyl aminomalonate. (A) $H_2(SB + Q)^-$, form protonated on the phosphate group; $H(SB + Q)^{2-}$, form with neutral ring; $(SB + Q)^{3-}$, high pH unprotonated form, presumably largely resonance forms IIIB and IIIC of Scheme I. (B) Form $(SB + Q)^{3-}$ resolved with lognormal curves.

Solutions of PLP or *N*-methyl-PLP + diethyl aminomalonate have also been studied by proton NMR spectroscopy. The reaction mixtures contained the aldehyde at concentrations of 0.5–2 mM and usually with diethyl aminomalonate in 2–5-fold excess. The spectra were relatively simple and some of them could be interpreted as indicating a mixture of only two components: aldehyde and a Schiff base or quinonoid species. The UV–visible spectra observed with a 1.0-mm path length on the same samples used for NMR spectroscopy were consistent with this conclusion. The chemical shifts relative to 3-(trimethylsilyl)-1-propanesulfonic acid (DSS) are given

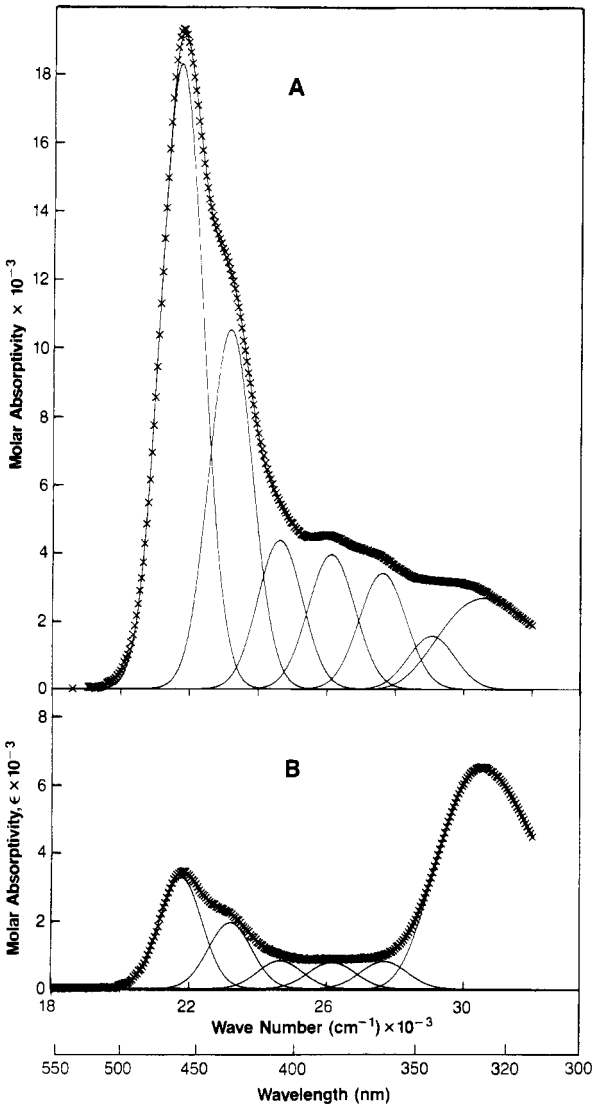


FIGURE 6: Analysis of spectral scans from Figure 2 (A) at 1.2 min and (B) at 122 min using Gaussian bands and lognormal curves.

Table II: Proton NMR Resonances Observed for Mixtures of Pyridoxal Derivatives and Diethyl Aminomalonate[a] in D_2O, Apparent pH 6–7

position in molecule	pyridoxal[b] SB	PLP free	PLP SB + Q	*N*-methyl-PLP free	*N*-methyl-PLP SB + Q
4′-H	9.00	10.44	9.18	10.41	9.20
aldehyde hydrate		6.25		6.22	
aldol 1		5.51			
aldol 2		5.58			
alanine SB[c]	8.98				
6-H	7.97	7.77	7.68	7.77	7.81
aldol 1		7.74			
aldol 2		7.72			
alanine SB[c]	7.58				
N–CH_3				4.14	4.12
2′-CH_3	2.38	2.48	2.56	2.62	2.59
alanine SB[c]	2.40				

[a] Diethyl aminomalonate in D_2O shows a triplet at 1.30 ppm and a quartet at 4.30 ppm. [b] From Sala et al. (1987). The solvent was a mixture of deuteriated dimethyl sulfoxide (DMSO-d_6) and D_2O. In DMSO-d_6 alone the α hydrogen of Schiff base was seen at 5.37 ppm. [c] Positions for the Schiff base of PLP + alanine have been added for comparison.

in Table II. For 2 mM PLP and 4 mM diethyl aminomalonate at an apparent pH of 6.2 the formation constant of 236 M^{-1} and pK_a values given in eq 3 predict a ratio of [SB + Q] to [PLP] of about 0.9. This is very close to the ratio

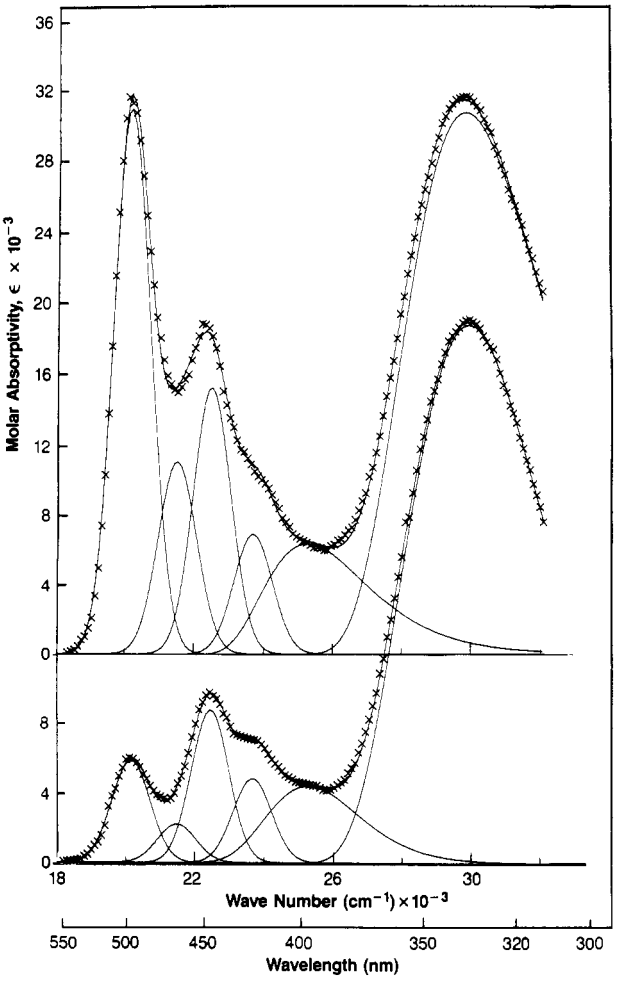


FIGURE 7: Effect of addition of KOH on the spectrum of 8.9×10^{-5} M pyridoxal + 8.9×10^{-4} M alanine ethyl ester + 2.2×10^{-4} M $Al(NO_3)_2$ in methanol as described by Matsumoto and Matsushima (1974). Both curves were obtained by extrapolation to zero time. The apparent pH values measured with a glass electrode are (top curve) 6.3 and (bottom curve) 7.0. The latter was obtained after addition of a small amount of KOH. The spectra were resolved with lognormal curves. A spectrum for pH 6.7 was also fitted equally well. The band parameters are given in Table I. The first four bands from the left in each spectrum are Gaussian, and the other two are lognormal curves.

of the 4′-H peak areas seen in the NMR spectrum. The observed chemical shifts are similar to those reported by Sala et al. (1987) for pyridoxal + diethyl aminomalonate in deuteriated dimethyl sulfoxide or deuteriated dimethyl sulfoxide plus D_2O. Thus, the data support the proposition that the "Schiff base" seen in the NMR spectrum is the same as the quinonoid species giving rise to the intense 463-nm band.

A complication was the rapid development in some samples of two additional components that appear to be aldol condensation products. These are designated aldol 1 and aldol 2 in Table II. They became the major components in samples prepared from one batch of diethyl aminomalonate, perhaps because of the presence of some impurity that catalyzed their formation. However, their content was almost indetectable in the solutions for which data are reported here.

Discussion

Our goal in this study was to establish the state of protonation and pK_a values for quinonoid species derived from PLP in enzymes or in nonenzymic systems. The four enzymic quinonoid species (Figure 1) have similar spectra with vibrational subbands from 1020 to 1110 cm^{-1} apart. They all appear to be in pH-independent equilibria with Schiff base

forms having the state of protonation of the ring shown in Scheme I. The fact that the quinonoid species from *O*-methyl-PLP and L-aspartate has a spectrum very similar to those given by PLP (Table I) suggests strongly (Chen et al., 1987) that the chelated proton in quinonoid III of Scheme I is on the phenolic oxygen rather than on the imine nitrogen. Previously Matsumoto and Matsushima (1972, 1974) had suggested that migration of the proton from the imine nitrogen of the Schiff base to the phenolic $-O^-$ accompanies formation of the quinonoid structure. Replacement of the –OH by $-OAl^{2+}$ could provide the observed stabilization of the quinonoid forms in methanol by aluminum salts. Ledbetter et al. (1979) observed that laser-induced formation of a quinonoid band was favored in an Al^{3+}–phenolate complex of Schiff bases of pyridoxal plus amines but not when chelation of the metal occurred.

The characteristic shape of the quinonoid adsorption bands is reminiscent of those of cyanine dyes and various other elongated chromophores (Malhotra & Whiting, 1960). It is probable that the prominent shoulder on the high-energy side of the highest peak represents vibrational structure. Such spectra can sometimes be approximated as the sum of a series of Gaussian bands whose relative heights follow a Poisson distribution (Gal et al., 1973). We have shown previously (Metzler et al., 1987; Chen et al., 1987) that the 491-nm absorption band of the *erythro*-3-hydroxyaspartate complex of cytosolic aspartate aminotransferase can be represented as the sum of a series of three nearly Gaussian lognormal curves of constant width and skewness and placed at intervals of about 1200 cm^{-1}. This spacing of the subbands may be related to the 1238-cm^{-1} frequency seen in the coherent anti-Stokes Raman spectrum of the aspartate aminotransferases–*erythro*-3-hydroxyaspartate complex (Benecky et al., 1985).

Our present results show that the use of Gaussian (skewness = 1.0) or nearly Gaussian curves will give an extremely good fit for all of the quinonoid bands, whether of enzyme complexes, diethyl aminomalonate derivatives, or aluminum complexes of alanine ethyl ester and pyridoxal. The narrow Gaussian bands have been combined with lognormal curves for the envelope of broader absorption bands to give complete descriptions of the spectra (Figures 1, 3B, 4B, 6, and 7; Table I).

An important question is whether or not the nonenzymic quinonoid species are chemically the same as those present in enzymes. The similarities are obvious: a narrow structured absorption band of high intensity and long wavelength. However, there are differences. The spacing between the component vibrational bands is somewhat greater (1330–1430 cm^{-1}) for the nonenzymic spectra. The 1330-cm^{-1} spacing seen for the methanolic ethyl alaninate–pyridoxal–Al^{3+} system corresponds closely to the intense 1332-cm^{-1} frequency of the coherent anti-Stokes Raman scattering on a similar phenylalanine ethyl ester system (Benecky et al., 1985). The wavelengths of maximum absorption are from 480 to 500 nm for enzyme spectra but about 460 nm for the intense bands from diethyl aminomalonate. In addition, the diethyl aminomalonate systems contain a weaker band system on the high-energy (low-wavelength) side that appears to be absent in enzymes.

One of the strongest arguments in favor of the quinonoid structure is the high intensity of the absorption bands. The molar areas of typical Schiff bases of pyridoxal phosphate, whether free or in enzymes, are about 350–400 Mm/mol, while that of an enzymic quinonoid species has been estimated as 698 Mm/mol (Metzler & Metzler, 1987). The areas of the six Gaussian subbands in the diethyl aminomalonate–PLP spectra are estimated as over 1000 Mm/mol.

Both the greater intensity of the quinonoid bands from diethyl aminomalonate and the greater width than for those from ethyl alaninate may be a result of the presence of the second ester group. This may also account for the fact that the wavelength of maximum absorption is at a higher energy for the PLP–diethyl aminomalonate complex (468 nm in methanol) than for the Al^{3+} complex of pyridoxal and alanine ethyl ester (486 nm in methanol). The opposite orientation of the two-electron-withdrawing ester grouping in the diethyl aminomalonate derivatives may cause this shift to higher energy. In terms of energy, however, the difference is small. The energy of excitation is only 11 kJ/mol less for photons of 480-nm wavelength than for photons of a 460-nm wavelength. Some differences in positions of quinonoid bands in enzymes or nonenzymic systems may result from "solvent effects". Thus, the difference in peak position for the PLP–diethyl aminomalonate quinonoid between water and methanol is 11 nm (Table I).

The alternative possibility, suggested by Sala et al. (1987), is that the intense 460-nm band with diethyl aminomalonate represents not a quinonoid species but a Schiff base. The strongest argument in favor of a Schiff base structure is that a precipitated quinonoid compound, when dissolved in deuteriated dimethyl sulfoxide, gave the proton NMR spectrum expected for a Schiff base. Thus, the C^4–H resonance was found at 9.06 ppm. By using PLP and *N*-methyl-PLP rather than pyridoxal, we were able to run NMR spectra on solutions of a variety of compositions and pH values in D_2O. We also found the NMR spectra to resemble closely those of Schiff bases. For example, at pH 6.2, a solution of 2 mM PLP and 4 mM diethyl aminomalonate appeared to contain only two major components: PLP (C^4–H at 10.4 ppm) and a component with the C^4–H resonance at 9.18 ppm. However, we suggest that this latter resonance may arise from a rapidly equilibrating mixture of Schiff base plus quinonoid form. We find it difficult to predict the NMR spectrum of the quinonoid species. It could be very close to that of the Schiff base. We thought that the NMR resonance of the *N*-methyl group of the quinonoid form derived from *N*-methyl-PLP might undergo a strong upfield shift from that in the free aldehyde. However, this was not observed. A possible explanation is that the quinonoid character of the intermediate is seen mostly in the photoexcited state and that in the ground state a more localized carbanionic structure, as proposed by Martell (1982), predominates.

If we accept the structure of the PLP–diethyl aminomalonate complexes as quinonoid IV of Scheme II, we must ask what causes the weaker bands in the 360-nm region. We suggest that these may represent tautomer V, in which the chelated proton has migrated back to the imine nitrogen. Similarly, tautomerism between VI and VII could occur in the higher pH form shown in Figure 5. During the experiment depicted in Figure 2 the ratio of the areas of the higher energy transition (Gaussian subbands 4–6) to the areas of the first three subbands was constant through the first 122 min of decay. If we assume that both tautomers IV and V (Scheme II) have the same molar area, the ratio of V (bands 4–6)/IV (bands 1–3) = 0.27.

An alternative possibility is that the second set of subbands represents not a tautomer but an additional electronic transition. However, the O^3-methyl-PLP-containing quinonoid complex (Figure 1B) has no absorption that could be attributed to a second electronic transition. Tautomerism is also im-

possible for this species. The Al^{3+} complex of pyridoxal and alanine ethyl ester in methanol (Table I) also has no proton that could participate in such tautomerism. It does have a weak structured band but a considerably higher energy relative to that of the major band.

The band at 327 nm in the 1.25-min spectrum of Figure 6 cannot be PMP, even though the latter compound appears later at this same position. It could very reasonably represent a higher energy transition belonging to the quinonoid species. A corresponding band appears to be present in the spectrum of the *erythro*-3-hydroxyaspartate complex of aspartate aminotransferase (Figure 1A).

The reason for the 10-nm difference in the position of the lowest energy band for the Al^{3+}–pyridoxal–alanine ethyl ester complex alone and in the presence of excess alanine ethyl ester (Table I) is not obvious. Perhaps a molecular complex is formed at the higher concentration ratio. We found nearly the same values for the positions and widths of the Gaussian and lognormal curves used to resolve both spectra of Figure 7 as well as one at an intermediate pH. We assumed that each of the two most intense bands was accompanied by a weaker band appropriately spaced to represent vibrational structure (Table I). Two of the resolved spectra are shown in Figure 7. It is clear that our representation is reasonable. The spacing between the major bands is 2270 cm^{-1}. Although this is greater than the 810-cm^{-1} difference between the positions of the first subbands of forms $H(SB + Q)^{2-}$ and $(SB + Q)^{3-}$ of eq 3, it is still reasonable to propose that the spectral changes observed with pH in Figure 7 are related to that seen in Figure 5. It is also possible that quinonoid bands with maxima around 400 nm in enzymes may represent dissociated structures such as VI of Scheme II.

We are unable yet to say whether there is a significant amount of Schiff base in equilibrium with the neutral quinonoid forms within the complex $H(SB + Q)^{2-}$ of eq 3 or within $(SB + Q)^{2-}$ of eq 1. However, K_Q is probably quite high. The pK_a of diethyl aminomalonate is 13.3, about 11 units lower than that of ethyl acetate (Pearson & Dillon, 1953). The pK_a of the α CH of the most protonated form of the Schiff base of alanine with 3-hydroxypyridine-4-aldehyde has been estimated by Dixon and Bruice (1973) as approximately 9. We may expect that the pK_a for the corresponding Schiff base of diethyl aminomalonate will be several units lower than this as a result of addition of the second electron-withdrawing substituent. The same should be true for Schiff base form H_2SB^- of eq 3. For this to be true K_Q must be quite high (>100) or the pK_a of the protonated imine group of H_2SB^- must be unexpectedly low.

Acknowledgments

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Chemical Modification of Prothrombin Fragment 1: Documentation of Sequential, Two-Stage Loss of Protein Function†

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ABSTRACT: The amino groups of prothrombin fragment 1 (amino acids 1–156 of prothrombin) were derivatized by acetylation, amidination, and reductive methylation. Conditions that caused complete acetylation of protein amino groups produced a fragment 1 derivative which no longer displayed a metal ion dependent intrinsic fluorescence change and had lost its membrane binding capability as well. However, when derivatized in the presence of calcium ions, extensive acetylation yielded a product that underwent protein fluorescence quenching at metal ion concentrations similar to those observed for the native protein. This derivative bound to membranes in a calcium-dependent manner with only a small reduction in affinity. Several results showed the existence of a partially functional protein that was characterized by a high degree of calcium-dependent protein fluorescence quenching but which had a requirement for 10-fold higher calcium concentration. This derivative was produced by partial acetylation (>3 equiv) of metal-free protein. This partially acetylated protein had greatly diminished membrane binding. The calcium-protected amino group, therefore, was among the most reactive acetylation sites in the metal-free protein. The second site, responsible for abolishing all metal ion induced fluorescence change, was resistant to acetylation and became derivatized at the last stages of amino group acetylation. The second site did not function as a substitute for the first site. That is, both sites were shown to be essential for full protein function so that calcium actually protected both sites from acetylation. The second site, but not the first site, could undergo deacetylation under alkaline conditions and mild heat (pH 10, 50 °C) or with hydroxylamine and mild heat (0.2 M, 50 °C). Thus, the fully acetylated protein could be returned to its intermediate state of function (high calcium requirement, low membrane binding) by these treatments. Amidination and reductive methylation of fragment 1 produced derivatives which were similar to the partially acetylated protein. These derivatives underwent protein fluorescence quenching which required 10-fold more calcium than native protein and had greatly reduced membrane affinity. This indicated that these subtle changes abolished the function of the first site but did not alter the function of the second site.

Prothrombin, a plasma glycoprotein, is one of a group of proteins which requires vitamin K for the posttranslational carboxylation of glutamyl residues. The resulting calcium binding protein, which contains 10 γ-carboxyglutamyl (Gla)[1] residues in its amino-terminal domain, is capable of calcium-dependent membrane binding [reviewed in Nelsestuen (1984)]. Prothrombin fragment 1 (amino acids 1–156 of prothrombin) contains all of the Gla residues of prothrombin and has been used as a model peptide for calcium and membrane binding studies of vitamin K dependent proteins [Gitel et al., 1973; reviewed in Nelsestuen (1984)]. Recent X-ray crystallographic analyses of fragment 1 provide the potential for a detailed understanding of calcium ion binding to a Gla-containing protein (Tulinsky & Park, 1986). Relatively little is currently known about the precise metal ion binding sites in fragment 1. Loss of as few as two Gla residues results in considerable loss of protein function (Malhotra et al., 1985; Borowski et al., 1985; Wright et al., 1986). This has greatly restricted the ability for modification of Gla residues as an approach to understanding the metal binding functions of fragment 1.

The present investigations were initiated to expand our understanding of the fragment 1 structure and to identify specific groups important to the various functions of fragment 1. These approaches may also offer methods of producing chemically modified proteins which allow insertion of spectroscopic probes at precise sites in the protein. The results provided direct chemical evidence for involvement of amino groups in the calcium and membrane binding activities of prothrombin fragment 1. Two essential sites were found that were sensitive to acetylation. Both were protected from ace-

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; fragment 1, amino acids 1–156 of the amino terminus of bovine prothrombin; EDTA, ethylenediaminetetraacetic acid; TNBS, trinitrobenzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TSP, (trimethylsilyl)propionate sodium salt.

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