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Studies of 6-Fluoropyridoxal and 6-Fluoropyridoxamine 5′-Phosphates in Cytosolic Aspartate Aminotransferase†

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ABSTRACT: The chemical and spectroscopic properties of 6-fluoropyridoxal 5′-phosphate, of its Schiff base with valine, and of 6-fluoropyridoxamine 5′-phosphate have been investigated. The modified coenzymes have also been combined with the apo form of cytosolic aspartate aminotransferase, and the properties of the resulting enzymes and of their complexes with substrates and inhibitors have been recorded. Although the presence of the 6-fluoro substituent reduces the basicity of the ring nitrogen over 10000-fold, the modified coenzymes bind predominately in their dipolar ionic ring forms as do the natural coenzymes. Enzyme containing the modified coenzymes binds substrates and dicarboxylate inhibitors normally and has about 42% of the catalytic activity of the native enzyme. The fluorine nucleus provides a convenient NMR probe that is sensitive to changes in the state of protonation of both the ring nitrogen and the imine or the -OH group of free enzyme and of complexes with substrates or inhibitors. The NMR measurements show that the ring nitrogen of bound 6-fluoropyridoxamine phosphate is protonated at pH 7 or below but becomes deprotonated at high pH around a pK_a of 8.2. The bound 6-fluoropyridoxal phosphate, which exists as a Schiff base with a dipolar ionic ring at high pH, becomes protonated with a pK_a of ~7.1, corresponding to the pK_a of ~6.4 in the native enzyme. Below this pK_a a single ^{19}F resonance is seen, but there are two light absorption bands corresponding to ketoenamine and enolimine tautomers of the Schiff base. The tautomeric ratio is altered markedly upon binding of dicarboxylate inhibitors. From the chemical shift values, we conclude that during the rapid tautomerization a proton is synchronously moved from the ring nitrogen (in the ketoenamine) onto the aspartate-222 carboxylate (in the enolimine). The possible implications for catalysis are discussed.

The function of pyridoxal 5′-phosphate (PLP)[1] in its catalysis of reactions of amino acids is to provide an electron-accepting center that permits cleavage of C–H or C–C bonds within the substrate. This function makes use of the powerful electron-withdrawing action of the N-protonated pyridine ring (**1**). However, this electron withdrawal is modulated by the donation of electrons into the ring from the unprotonated phenolic

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; 6-FPLP, 6-fluoropyridoxal 5′-phosphate; 6-FPMP, 6-fluoropyridoxamine 5′-phosphate; E-FPLP and E-FPMP, complexes of cytosolic apo-aspartate aminotransferase with 6-FPLP and 6-FPMP; Tris, tris(hydroxymethyl)aminomethane.

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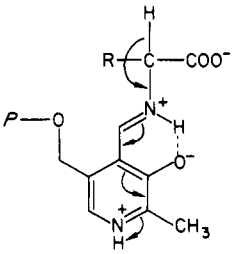

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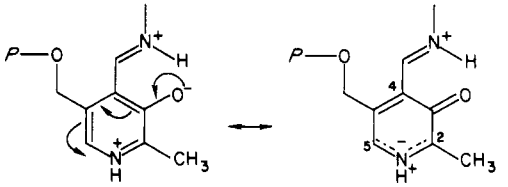

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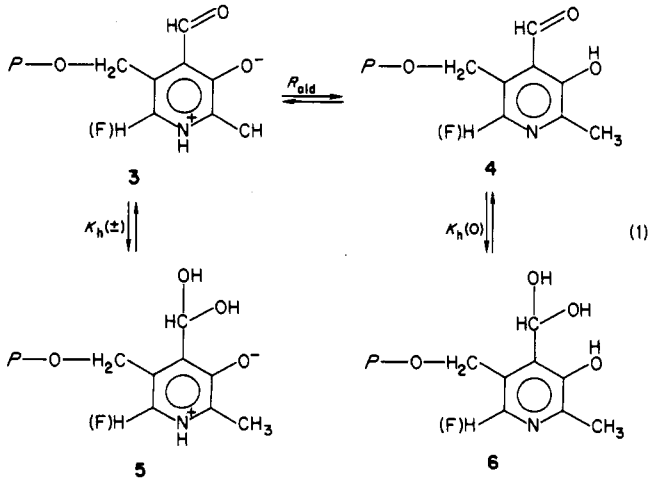
oxygen (**2**). This electron donation neutralizes the charge on the ring by placing electron density on C-2 and C-6. For simple 3-hydroxypyridines this can raise the pK_a of the ring nitrogen by 3 or more units. This effect is also influenced greatly by the presence or absence of an imine (Schiff base) group in the 4-position and by the state of protonation of the imine nitrogen. Adding to the complexity is the fact that Schiff bases of PLP can tautomerize readily. For example, a proton can move from the imine nitrogen to the adjacent phenolic oxygen (Heinert & Martell, 1963; Metzler et al., 1980; Kallen et al., 1985).

The chemical shift of the 6-H of the coenzyme in proton NMR spectra is an indicator of the electron density at C-6 and is affected by the protonation of both the ring nitrogen and the phenolate oxygen (Korytnyk & Ahrens, 1970). It is difficult to locate the NMR resonance for this proton in spectra of enzymes, but a fluorine-19 nucleus, in the same position, provides a sensitive NMR probe that can be observed in the protein-bound coenzyme (Chang & Graves 1982, 1985; Scott et al., 1983, 1984). A complication arises from the fact that 6-fluoropyridoxal 5′-phosphate (6-FPLP) exists in aqueous solution almost exclusively as the nondipolar ionic tautomer **4** whereas PLP itself is mainly the dipolar ion **3**. These two forms, as well as their covalent hydrates, **5** and **6**, are present in an equilibrium mixture (eq 1). Similarly, 6-fluoro-



(1)

pyridoxamine 5′-phosphate (6-FPMP) exists exclusively with a nondipolar ionic ring whereas the natural coenzyme pyridoxamine phosphate (PMP) is almost entirely dipolar ionic. Since the apo form of aspartate aminotransferases is known to bind the dipolar ionic form **3** of PLP to give a dipolar ionic Schiff base (Figure 1) and PMP also binds as the dipolar ion, it seemed unlikely that 6-FPLP and 6-FPMP would bind normally to this apoenzyme. On the other hand, we expected that 6-FPLP might bind well to the apoenzyme of glycogen phosphorylase, which forms a Schiff base with tautomer **4**. As shown by Chang & Graves (1985), 6-FPLP does bind to apo-rabbit muscle glycogen phosphorylase *b* and has 28% of the activity observed with PLP. However, both 6-FPLP and 6-FPMP also bind to the apoenzyme of cytosolic aspartate aminotransferase from pig hearts. The resultant enzyme is nearly half as active as native enzyme, and the ^{19}F nucleus in the 6-position provides a readily measurable NMR signal that responds to changes in pH and to reactions with substrates and inhibitors (Scott et al., 1984). This paper provides additional information about these reactions and about the properties of 6-FPMP, 6-FPLP, and its Schiff bases.

Materials and Methods

The 6-FPLP was synthesized as described by Chang & Graves (1985). The molar absorptivity in 0.1 M HCl (3.50×10^3 M^{-1} cm^{-1} at 355 nm) was evaluated on a highly purified sample from the absorption spectrum and the phosphate content. The 6-FPMP was prepared by dissolving 6-FPLP (0.4 mg) in 30% aqueous NH_4OH. A small amount of solid $NaBH_4$ was added to this solution. The resulting mixture was concentrated by lyophilization and applied to a Bio-Gel P-2 column (1 × 60 cm). The column was washed with 0.01 M formic acid, and fractions absorbing at 290 nm were pooled (Chang & Graves, 1985).

The α subform of the cytoplasmic isoenzyme of aspartate aminotransferase of pig hearts was prepared by the method of Jenkins et al. (1959) as modified by Martinez-Carrion et al. (1967; method A). The apoenzyme was obtained by the procedure of Scardi et al. (1963) as modified by Furbish et al. (1969). The 6-FPLP-containing enzyme was prepared by adding 1.2–1.5 mol of 6-FPLP/mol of apoenzyme active sites to an approximately 0.4 mM solution (18 mg/mL) of apoenzyme at pH 8.3 in 0.02 M Tris–acetate or triethanolamine–acetate buffer. The apoenzyme concentration was estimated with a molar extinction coefficient of 6.36×10^4 at 280 nm on the basis of the monomer concentration. (The protein is dimeric.) After 2 h the enzyme was dialyzed for 12–24 h with three changes of buffer at about 5 °C. Dialysis was continued for a similar period at pH 5.4 to remove a small amount of nonspecifically bound 6-FPLP.

UV–visible absorption spectra were recorded in digital form on a Cary 219 spectrophotometer interfaced to an Apple 2E computer or on a Cary 1501 spectrophotometer as described previously (Metzler et al., 1973). Data points were collected at regular intervals of 2 nm or of 200 cm^{-1}. Resolution with lognormal curves was accomplished as described previously (Metzler et al., 1973, 1980; Harris et al., 1976). Width and skewness values for minor bands were fixed at preselected values. An attempt was made to find an internally consistent set of band parameters that could be related directly to those of the 6-H compounds.

The ^{19}F NMR measurements were made on a Bruker WM-300 spectrometer at 282.4 MHz. Spectra were collected with a 50–90° pulse (15–30 μs) with 8K of data points and an acquisition time of 0.205 s. The relaxation delay was varied from 0.2 to 5.8 s. An exponential multiplication of 20 Hz was applied to the free induction decay before Fourier transformation. Peak shifts are in reference to external 20 mM trifluoroacetate, pH 6.8, as 0 ppm. This places fluorobenzene at −38.1 ppm. For calculation of line widths, 20 Hz was subtracted from the measured widths at half-height. No

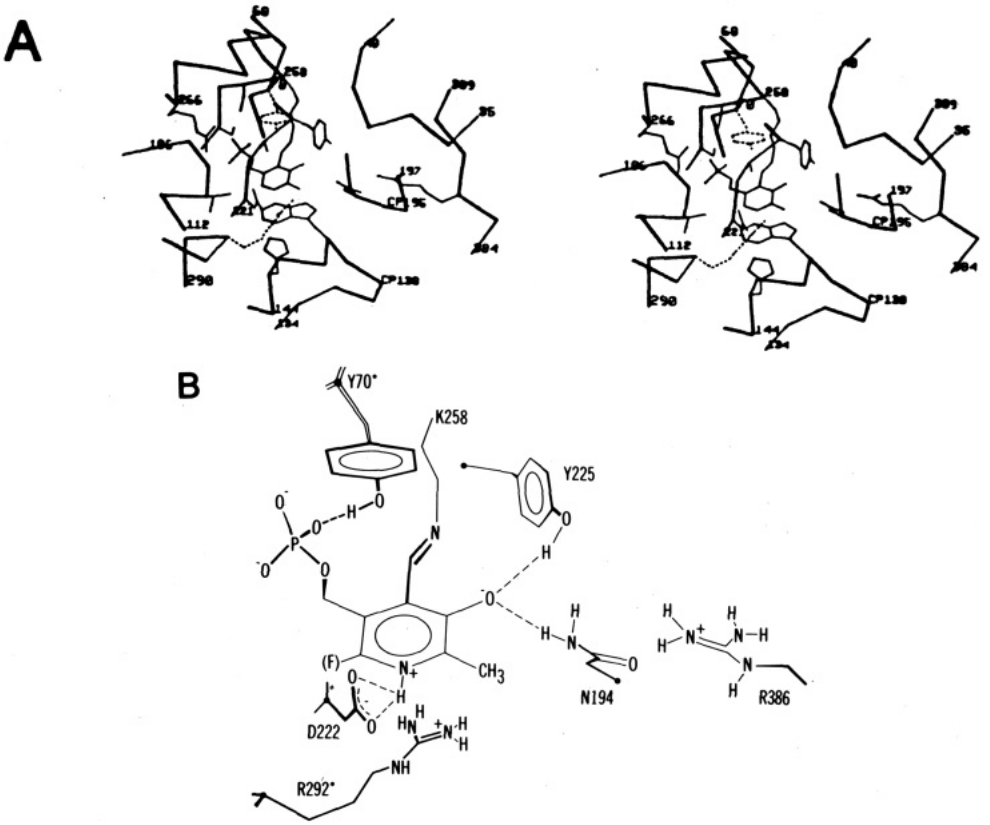


FIGURE 1: Active site of cytosolic aspartate aminotransferase of pig hearts as determined by X-ray crystallography (Arnone et al., 1985a): (A) stereo diagram showing coenzyme ring and some surrounding residues; (B) schematic drawing.

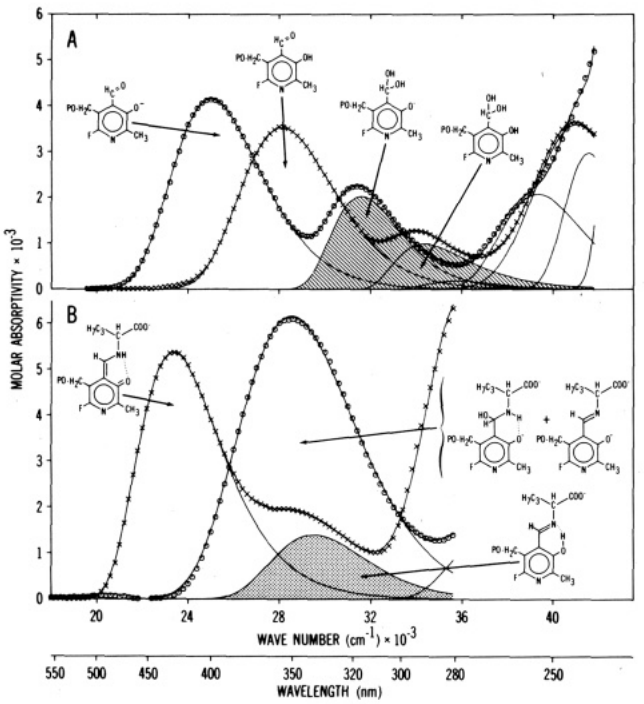


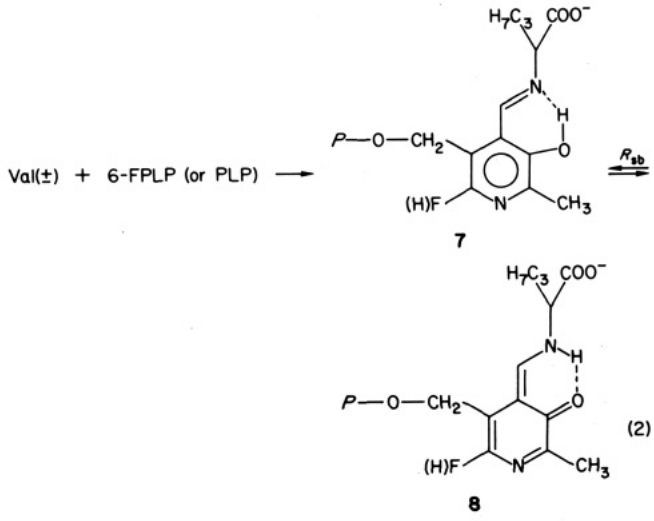
FIGURE 2: Absorption spectra of single ionic forms of 6-FPLP and of its Schiff base with valine. For each compound the spectra of forms with a neutral uncharged ring and with an anionic ring are shown. These were computed from a series of spectra at differing values of pH. (A) 6-FPLP; shaded bands represent covalent hydrate; (B) Schiff base; shaded band represents enolimine tautomer **7**.

broad-band decoupling of proton resonances was used.

RESULTS

Nonenzymatic Experiments. Electronic and ^{19}F NMR spectra were measured on free 6-FPMP, on 6-FPLP and its Schiff base with valine, and on some related compounds. Variations with pH and, for the Schiff base, with valine concentration were used to calculate the pK_a value and Schiff-base formation constant (Nagano & Metzler, 1967; Metzler et al., 1980). The spectra of some individual ionic forms are shown in Figures 2 and 3. The spectra have been analyzed by fitting with lognormal distribution curves. Although some of the bands are as much as 20% wider than those for the corresponding 6-H compounds, the procedure appears to be entirely satisfactory and permits a quantitative accounting of all of the area in complex spectra with overlapping bands. The positions of band maxima, apparent molar absorptivities, and estimated fractions of different species are available as supplementary material [see paragraph at end of paper regarding supplementary material; see also Scott (1985)]. The pK_a values are given in Table I together with those of some related compounds. Hydration ratios and Schiff-base formation constants are given in Table II and chemical shifts of NMR resonances in Table IV.

Valine (dipolar ion) reacts with anionic 6-FPLP (eq 2) to form the monoanionic Schiff base with a formation constant at 25 °C of $10^{3.44}$ M, identical with that with PLP itself (Metzler et al., 1980). The structures of the two Schiff bases are also similar. The two major light absorption bands at 345 and 426 nm (Figure 2) can be assigned to the enolimine tautomer **7** and the ketoenamine tautomer **8**, respectively. The ratio of these (R_{sb} = [enolimine]/[ketoenamine]) was varied by changing the solvent from H_2O up to 95% methanol, in which **7** is strongly favored as is indicated by an increase in the 345-nm absorption band and a drop in the 426-nm band (see Table III of supplementary material). R_{sb} was estimated by the previously described method (Metzler et al., 1973). In this method, the ratio of changes in areas of the absorption bands of two interconvertible forms when the solvent composition is changed is assumed equal to the ratio of the molar



areas, i.e., the areas for single ionic species. In this way the ratio of molar area of **7** to that of **8** was estimated as 0.78, and the molar areas were estimated as 239×10^6 and 305×10^6 m/mol for **7** and **8**, respectively. The unprotonated form of the Schiff base has an unusually broad absorption band, which is consistent with the presence of both carbinolamine and Schiff base in nearly equal amounts as is indicated by ^{19}F NMR (Figure 6B, Table IV). As with the Schiff bases of PLP itself (Metzler et al., 1980; Kallen et al., 1984), the pK_a of the chelated proton in the mixture of **7** and **8** is >12.

A preliminary test of the nonenzymatic reactivity of 6-FPLP with an amino acid was made as follows. A solution containing 1 mM 6-FPLP, 111 mM L-glutamate, and 1 mM $KAl(SO_4)_2$, pH 5.0 (Metzler & Snell, 1952), in a closed 10-mm fused silica cuvette containing a 9.5-mm silica spacer (to give a path length of 0.50 mm) was held at 42 °C for a period of 11 h and subsequently at room temperature for 21 days. A control was run with PLP instead of 6-FPLP. The UV–visible absorption spectrum was recorded at various times. Over a period of 3 h the spectrum of the PLP-containing control solution changed rapidly to give a strong PMP band at 333 nm. However, the 6-FPLP-containing solution underwent an insignificant amount of spectral change even after 8 days. We conclude that the reactivity of 6-FPLP in nonenzymatic reactions is very much less than that of the natural coenzyme.

Interaction with Aspartate Aminotransferase. Binding of 6-FPLP to apo-aspartate aminotransferase within the pH range 6–8 is rapid and tight. After incubation of ~0.1 mM apoenzyme with a 10–20% excess of the analogue, the remaining free 6-FPLP can be dialyzed out. Prolonged dialysis (up to 100 h at pH 8.3 and 50 h at pH 5.4) then causes little or no additional loss of the fluorinated coenzyme. However, in the crystalline state at pH 5.4 a major fraction of the 6-FPLP was lost from one subunit [subunit 1; see Arnone et al. (1982, 1984, 1985) and Metzler et al. (1982)] and a lesser amount from the other (A. Arnone, private communication). Thus, the binding is less tight than for PLP itself. The binding of 6-FPLP to apoenzyme was done under conditions that provide for over 95% reactivation of apoenzyme by PLP.

The absorption spectrum of the 6-FPLP-containing enzyme (E–FPLP) is shown in Figure 4 together with that of the native enzyme. At pH 5.4 there are again two absorption bands, one at 345 nm and a weaker one at 447 nm. We believe that as with the free Schiff bases these correspond to the tautomers **7** and **8**, respectively. However, it is not immediately clear whether the ring nitrogen is protonated or whether the proton normally on the ring has been transferred wholly or in part

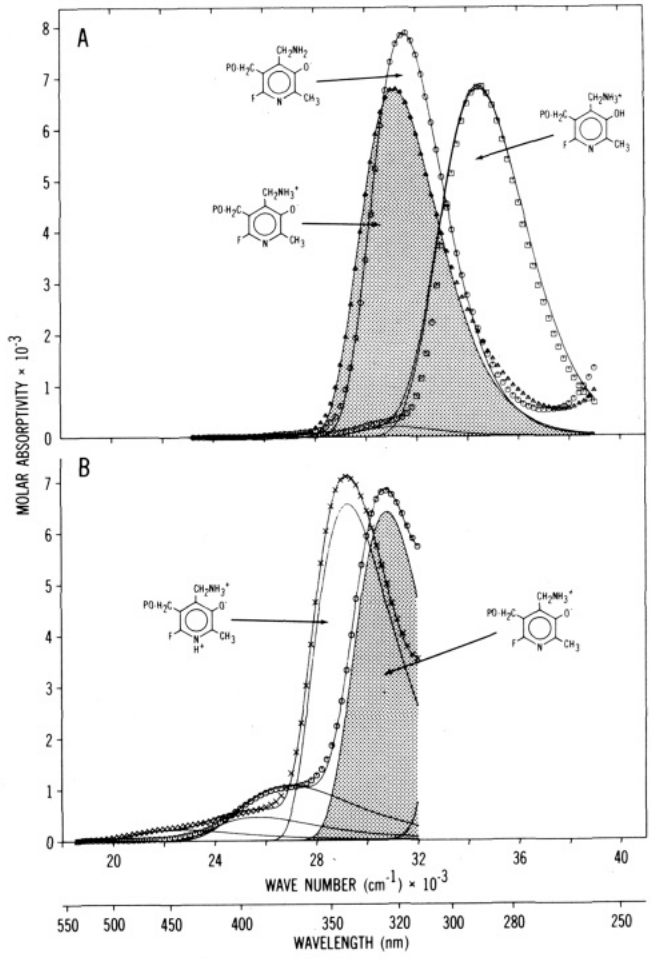


FIGURE 3: Absorption spectra of individual ionic forms of 6-FPMP calculated from a series of spectra at various values of pH: (A) free compound; (B) bound to apo-aspartate aminotransferase. We believe that the shaded bands represent the dipolar ionic species with an unprotonated ring nitrogen.

to the Asp-222 carboxylate group (see Figure 1). X-ray diffraction studies (A. Arnone, private communication) confirm that Lys-258 provides the amino group for this Schiff base as in the native enzyme. The absorption spectrum of E–FPLP in 20 mM Tris–acetate buffers changes with a pK_a of 7.1 at 10 °C (Figure 4), the high-pH spectrum having a maximum at 375 nm, appropriate for a dipolar ionic Schiff base as in the native enzyme (Figure 1). The low value of the pK_a, which is, however, somewhat higher than that for native enzyme (about 6.4 in similar buffers), is also suggestive of a dipolar ionic coenzyme ring.

The apparent pK_a of the coenzyme of native enzyme is strongly dependent upon the anion concentration (Boyde, 1968; Bergami et al., 1968; Giannini et al., 1976; Jenkins, 1980; Jenkins & Fonda, 1984), shifting from as low as 5.25 at very low ion concentrations (Braunstein, 1973) to about 6.8 upon saturation with a monovalent anion such as chloride or acetate. The pK_a of the E–FPLP complex is likewise sensitive to the anion concentration and in the presence of 0.5 M sodium acetate is raised to ~7.7 at 10 °C.

Dicarboxylates such as 2-oxoglutarate bind tightly to the PLP form of aspartate aminotransferase, causing a small bathochromic shift in the 430-nm band and an increase in the pK_a of the enzyme to about 8.7 (Jenkins, 1980; Fonda & Johnson, 1970; Jenkins & Fonda, 1984). With E–FPLP, glutarate, succinate, malate, and *meso*-tartrate also induce a bathochromic shift (Figure 4). The pK_a is raised to above 9 in the presence of 10 mM 2-oxoglutarate. The tautomeric ratio

Table I: Values of pK_a for 6-Fluoropyridoxamine 5′-Phosphate (6-FPMP), 6-Fluoropyridoxal 5′-Phosphate (6-FPLP), and Related Compounds

compd	method[a]	NH^+ (ring)	-OH	$=N^4H^+$ or $-NH_3^+$
4-fluorophenol	NMR		9.7	
6-fluoropyridoxine[b]	S	-0.2	8.2	
	NMR	0.05	8.3	
6-chloropyridoxine[b]	S	1.1	7.9	
6-bromopyridoxine[b]	S	1.0	7.7	
pyridoxine (microscopic constants)[c]	S			
cation		5.63		
neutral, nondipolar ionic			8.20	
6-FPMP	S		7.7	10.94
	NMR		7.45	
PMP[c]				
cation	S	~4.4		
neutral, nondipolar ionic form	S		~7.6	~8.4
6-FPLP	S	<0	7.8	
aldehyde[d]	NMR		8.1	
hydrate	NMR		7.9	
PLP[c]	S			
cation		~3.6		
neutral, nondipolar ionic form			~7.8	
Schiff bases with DL-valine				
and 6-FPLP	S			12.3
and PLP[e]	S	5.6		12.2
6-FPLP-containing cytosolic AspAT	S			~7.1
native AspAT[f]	S			~6.4
6-FPMP-containing AspAT,[g] pH 8.3	S	8.24		
	NMR	8.3		

[a] S, spectrophotometric; NMR, ^{19}F nuclear magnetic resonance. [b] Courtesy of C. M. Metzler and W. Korytnyk. [c] Microscopic dissociation constants, calculated from data of Metzler et al. (1973) and Harris et al. (1976); see also Kallen et al. (1984). [d] Chang & Graves (1985). [e] Metzler et al. (1980). [f] Varies from ~5.4 at low anion concentration to ~6.8 in high chloride or acetate. [g] In 20 mM Tris–acetate buffer.

Table II: Hydration Ratios of 6-Fluoropyridoxal 5′-Phosphate and Pyridoxal 5′-Phosphate and Formation Constants for Schiff Bases

	from NMR spectra (in D_2O)	from UV spectra
6-FPLP		
hydration ratios		
[hydrate]/[aldehyde][a]		
neutral ring	0.20	0.24
anion	0.52	0.44
formation constant of Schiff base with valine[b]		log K_f' = 3.44
hydration ratio [carbinolamine]/[Schiff base] for anion	~0.8	
PLP		
hydration ratios[c,d]		
cation	3.0, 4.1	3.2
dipolar ionic ring	0.20, 0.23	0.3
neutral ring		~0.3
anion	na, 0.03	0.09
formation constant of Schiff base with valine[b]		log K_f' = 3.44

[a] From ^{19}F NMR spectra. [b] K_f' = [monoprotonated Schiff base]/([valine(±)][unprotonated 6-FPLP or PLP]). [c] From proton NMR spectra, Ahrens et al. (1970; first value) and Kallen et al. (1985; second value); in D_2O. [d] From electronic absorption spectra; Harris et al. (1976). [e] From Metzler et al. (1980).

R_{sb} (=[enolimine]/[ketoenamine]) has been calculated for E–FPLP from the changes of the areas of the two bands with the dicarboxylate inhibitors. From these changes the ratio of molar areas of enolimine (345 nm) and ketoenamine (447 nm) bands was estimated as approximately 0.85, and the molar areas themselves as were estimated 289 × 10^6 and 339 × 10^6 m/mol, respectively. The ratio R_{sb} is decreased distinctly in the dicarboxylate complexes from about 2.0 in the free enzyme in 0.04 M acetate buffer, pH 5.4, to about 1.0 in the succinate and 0.64 in the L-malate complexes (see Figures 4A and 7B). However, little effect on this ratio is seen with 2-oxoglutarate or acetylenedicarboxylate. As with native enzyme the absorption spectrum of E–FPLP is affected only slightly by changes in temperature over the range 2–20 °C, the tautomeric ratio R_{sb} changing from 1.97 at 2 °C to 1.89 at 20 °C. Similar small changes with temperature occur for the succinate and glutarate complexes. Although these estimates of R_{sb} are only approximate, they do not rely on any assumption concerning the state of protonation of the ring nitrogen (see Discussion).

Table III: Kinetic Parameters of Apo-Cytosolic Aspartate Aminotransferase Reconstituted with either 6-FPLP or PLP at pH 8.3,[a] 25 °C

	+6-FPLP	+PLP	native holo-enzyme
V_{max} (s^{-1})	73	193	160,[b] 300[c]
K_m, 2-oxoglutarate (mM)	0.003	0.043	0.82,[b] 0.1[c]
K_m, aspartate (mM)	0.3	1.65	6.6,[b] 0.9[c]

[a] Buffered. [b] Kiick & Cook (1983). [c] Velick & Vavra (1962).

Enzymatic Activity. The 6-FPLP-containing aspartate aminotransferase has 42% as much activity as PLP when added back to apoenzymes, judging by the maximum velocity of the enzyme-catalyzed reaction of aspartate with 2-oxoglutarate. This velocity together with the four Michaelis constants were measured by the methods of Kiick & Cook (1983) and are given in Table III, where they are compared with the same parameters for native enzyme. It is evident that no major differences exist. However, it is not clear why our K_m values are so much lower than those of Kiick and Cook.

Electronic Spectra of Substrate Complexes. Native aspartate aminotransferase is converted by the inhibitory quasi-substrate 2-methylaspartate to a complex with absorption bands at 430 and 369 nm of approximately equal intensity (Fasella et al., 1966; Kallen et al., 1984). The 430-nm band has greatly diminished circular dichroism and is thought to represent the Schiff base of the inhibitor with the coenzyme. The 360-nm band may represent the first complex (the "Michaelis complex") formed prior to conversion by transimination to the "external Schiff base" with the inhibitor. With E–FPLP a similar complex is formed with 2-methylaspartate,

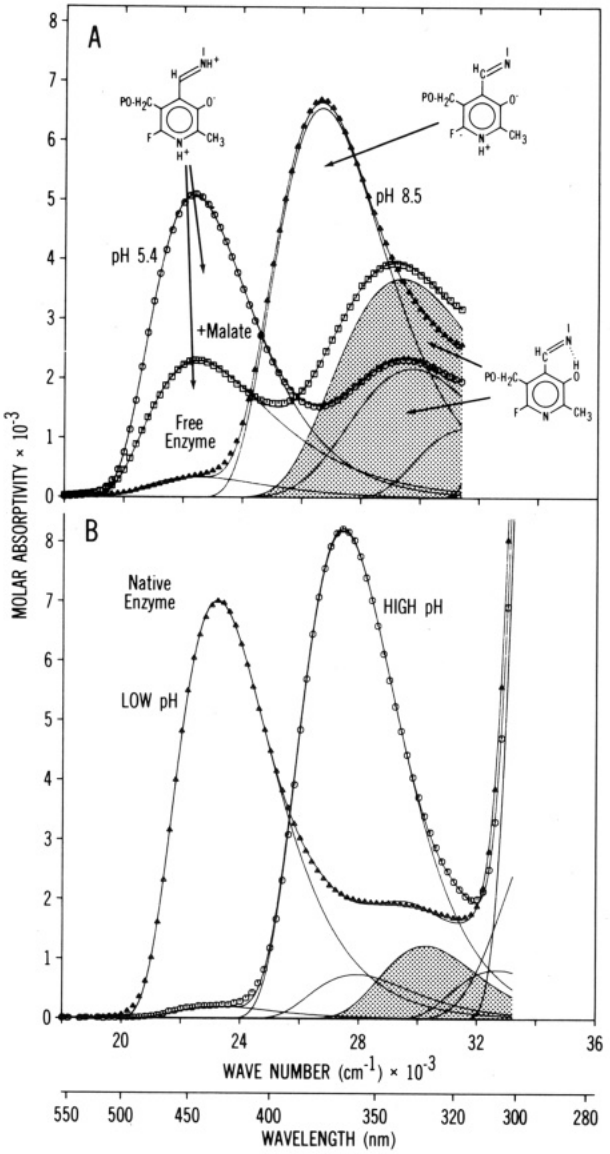


FIGURE 4: Absorption spectra of 6-FPLP bound to apo-aspartate aminotransferase compared with that of the native enzyme. (A) Spectra of bound 6-FPLP at low pH (~5.4) and high pH (~8.5), on both sides of the pK_a value of 7.1 (~6.4 for native enzyme). The spectrum of the malate complex at low pH is also shown. (B) Native enzyme. All spectra have been resolved with lognormal curves. The shaded bands are presumably enolimine tautomer.

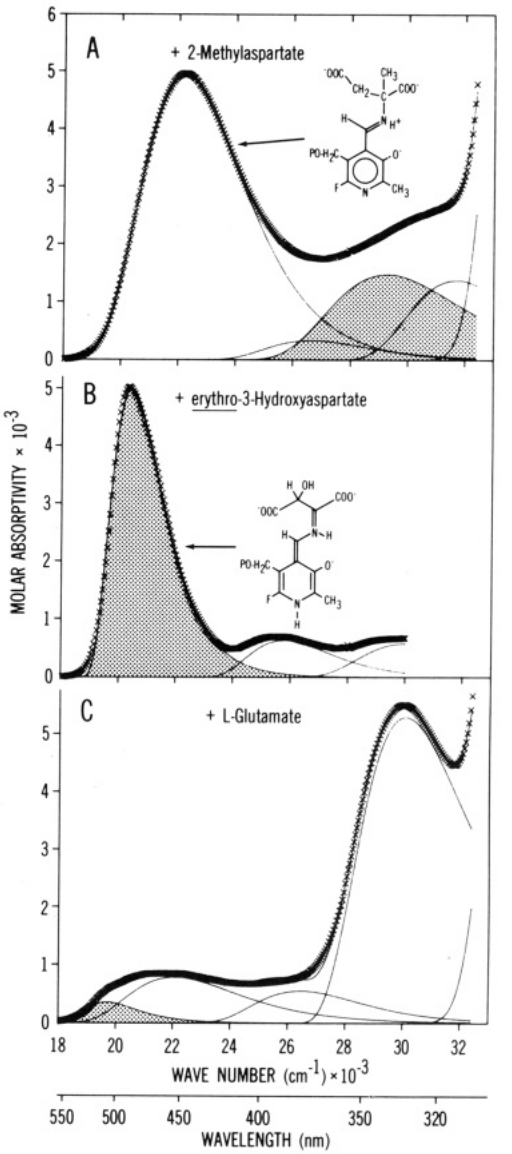


FIGURE 5: Spectra of 6-FPLP-containing aspartate aminotransferase after reaction with (A) 50 mM DL-2-methylaspartate, pH 8.3 (shaded band: enolimine tautomer), (B) 5 mM L-*erythro*-3-hydroxyaspartate, pH 5.4 [shaded band here and in (C) the quinonoid form], and (C) 50 mM L-glutamate, pH 8.3.

the dissociation constant being less than 1 mM at pH 8.3. However, with the fluorinated coenzyme the 451-nm ketoenamine band of the external Schiff base is the major band. The smaller band at ~340 nm (Figure 5) probably represents, in part, the corresponding enolimine. It is evident from attempts to resolve the spectrum with lognormal curves that additional absorption bands are also present. The smaller of these probably matches the high-pH dipolar ion band at 375 nm and corresponds to the much stronger 360-nm band seen in the 2-methylaspartate complex of native enzyme and attributed to the Michaelis complex. Another, at ~320 nm, may be a carbinolamine or a geminal diamine. From the areas of the four bands, we estimate the following percentages: ketoenamine **8**, 56%; 375-nm band (Michaelis complex), 5%; enolimine **7**, 19%; 320-nm band, 20%. The tautomeric ratio R drops from 2.0 in the free enzyme to ~0.46 in the complex.

The substrates L-glutamate and L-aspartate form complexes with E–FPLP having a more or less normal distribution of components including a 458-nm band and a small quinoid band at ~490 nm (Figure 5). With the very slow substrate *erythro*-3-hydroxyaspartate, a strong quinonoid band appears at 491 nm as well as bands at 390 and 330 nm. All of the spectra have been analyzed by fitting with lognormal curves; approximate molar areas and fractions estimated for different components are given in Table I of the supplementary material for several of these spectra.

Electronic Spectrum of E–FPMP. As with native enzyme the E–FPLP can be converted quantitatively to the E–FPMP form by treatment with L-cysteinesulfinate. However, the transformation is not instantaneous, a strong quinonoid band appearing at 491 nm and decaying over a period of <1 min. The spectrum of the enzyme resulting from addition of 1.2 equiv of cysteinesulfinate and passage through a gel filtration column is shown in Figure 3. Unlike that of native enzyme, this spectrum is pH-dependent, the absorption maximum shifting from 341 nm at low pH to 325 nm at high pH around a pK_a value of 8.24. When chemically synthesized 6-FPMP was recombined with apo-aspartate aminotransferase identically the same absorption spectra were generated. Addition of glutarate at pH 8.5 shifted the peak to 341 nm.

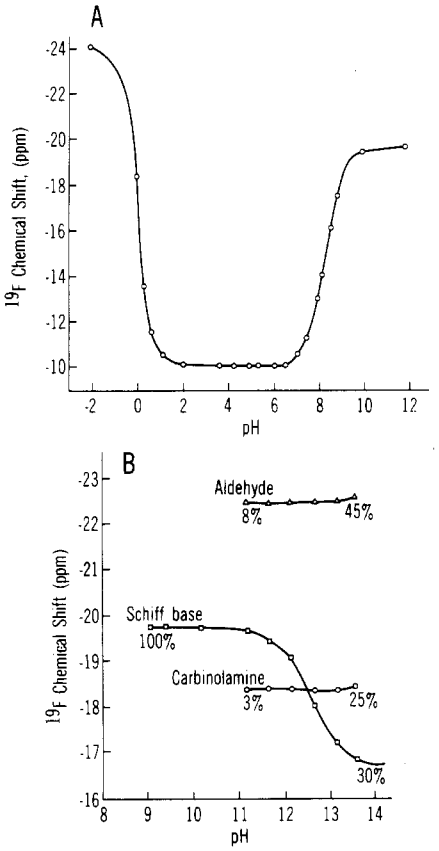


FIGURE 6: Fluorine-19 NMR titration curves: (A) for 6-fluoropyridoxine and (B) for the Schiff base of 6-FPLP and valine. The spectra were measured with 0.3 mM 6-FPLP and 0.1 M L-valine. Free 6-FPLP (aldehyde) is also present as is indicated. At high pH, there is a third resonance that we believe to be carbinolamine. The amounts (percentages) of the three components obtained by integration are shown at the high and low pH ends of each curve.

Fluorine NMR Spectra. The positions of the ^{19}F resonances as well as the band widths are given in Table IV for 6-fluoropyridoxine, for 6-FPMP, for 6-FPLP and its Schiff base with valine, for fluoride ion, and for some other reference compounds. For the benzenoid reference compounds, multiplets of narrow (~2 Hz in H_2O, 1 Hz in methanol) bands were observed in all cases. These showed the expected couplings to the hydrogen atoms present on the ring. For the pyridine derivatives, no coupling was seen but only a broader singlet. For the vitamin B_6 derivatives, band widths were about 8 Hz in all cases.

Dissociation of the phenolic hydroxyl of the fluorinated coenzymes produced the expected ("upfield") shift to lower frequencies. This amounted to −9.6, −10.7, −10.3, and −7.9 ppm for 6-fluoropyridoxine, 6-FPMP, 6-FPLP (aldehyde), and 6-FPLP (covalent hydrate), respectively. *o*-Fluoropyridines are so weakly basic that it is difficult to completely protonate the ring nitrogen. However, for 6-fluoropyridoxine, whose pK_a is about 0, it is clear that N-protonation gives a −14 ppm shift to lower frequency (Figure 6A). A shift of −10.2 ppm is seen when 2-fluoropyridine itself is placed in concentrated HCl (Table IV). However, it is probably not completely converted to the cation, even in this solvent.

For the Schiff base of 6-FPLP with valine a shift of ~3.2 ppm to a higher frequency accompanies dissociation of the proton from the imine nitrogen (Figure 6B). When the solvent for the Schiff base is changed from water to water–methanol mixtures of increasing methanol content, the ^{19}F resonance moves from −19.8 to −16.9 ppm in 95% methanol. When the ratio of enolimine to ketoenamine (calculated from data of

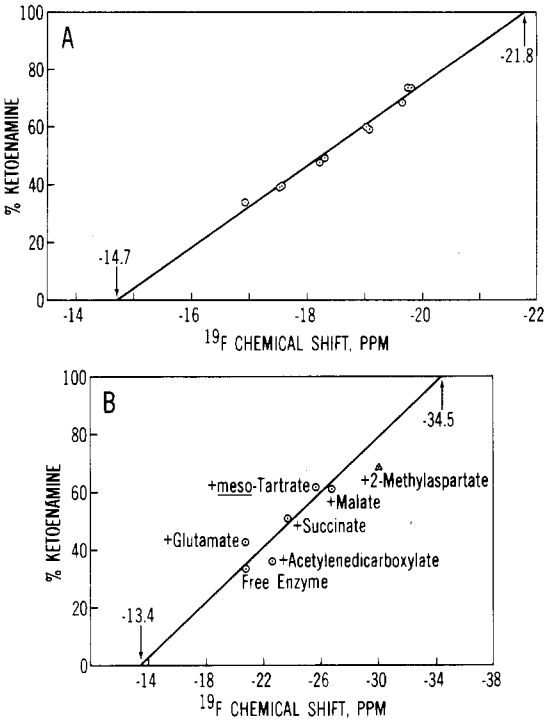


FIGURE 7: (A) ^{19}F chemical shift as a function of the fraction of ketoenamine (**8**) in the Schiff base of 6-FPLP + valine in water–methanol solvent mixtures. (B) ^{19}F chemical shift as a function of the fraction of the ketoenamine in 6-FPLP-containing aspartate aminotransferase and a series of its dicarboxylate complexes (dicarboxylate concentrations, 50 mM; acetylenedicarboxylate, 100 mM). In both cases the fraction of ketoenamine was estimated from the electronic absorption spectrum.

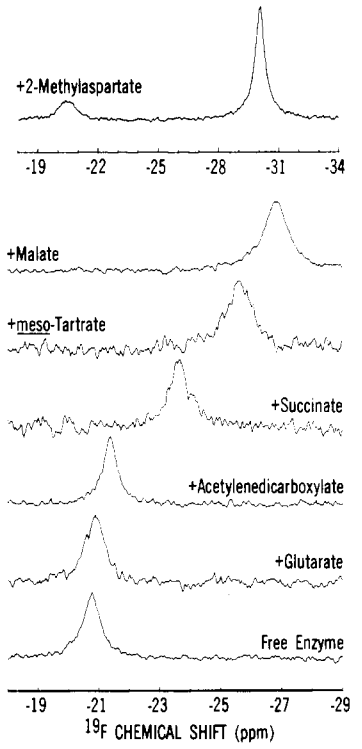


FIGURE 8: Fluorine-19 NMR spectra of 6-FPLP-containing aspartate aminotransferase and complexes with inhibitors. All are at pH 5.4.

Table II of the supplementary material) is plotted against the ^{19}F chemical shift, a nearly straight line relationship is observed (Figure 7A).

When bound to apo-aspartate aminotransferase, 6-FPLP has a single NMR resonance at −20.7 ppm (Figures 7B and

Table IV: Fluorine-19 NMR Data

	chemical shift, ppm relative to TFA[a]			
	in H_2O	other solvent	width (Hz)	T_1 (s)
fluoride ion, F^-	−44.1		2	
fluorobenzene	−38.2	−34.5 ($CHCl_3$)[b]	2	
2-fluoropyridine				
neutral	4.75	10.8 (dimethylformamide)[b]	18	
cation (in 12 M HCl)	−5.4		18	
4-fluorophenol				
neutral	−49.5	−49.0 (CCl_4)[c]	2	4.6
anion	56.6		2	
3-fluorobenzylamine				
cation	−37.2		2	
neutral	−38.5		2	
6-fluoropyridoxine				
cation	−24			
neutral	−10			1.3
anion	−19.6			
6-FPMP				
monocation (neutral ring)	−8.2			
dipolar ion (ring unprotonated)	−18.9			
6-FPLP				
neutral				
aldehyde	−12.1		8	
hydrate	−10.4		8	1.3
anion				
aldehyde	−22.4		8	
hydrate	−18.3		8	
Schiff base of 6-FPLP with valine				
neutral, enolimine + ketoenamine	−19.8		8	0.95
anion ring	−16.8			
anionic carbinolamine	−18.4			
E–FPLP				
low pH	−20.7		~200	0.55
high pH	−36.2		~200	0.77
complexes of E–FPLP				
+2-oxoglutarate, pH 5.4 or 8.3	−20.7		~120	
+2-methylaspartate, pH 5.4	−30.1		~80	0.6
minor band	−20.3			
+L-glutamate, pH 5.4 or 8.3	−32.0		~600	0.9
minor band	−20.5		280	0.9
+L-aspartate	−30.6		260	
+DL-*erythro*-3-hydroxyaspartate				
major band	−36.6		~100	0.7
minor band	−34.4		~100	1.3
+succinate	−23.68			0.45
+glutarate	−21.38			0.45
6-FPMP + apo-AspAT				
pH 8	−35.3		~200	
	−22.3			
generated in situ with cysteine sulfinate				
pH 5	−35.3		~200	0.9
pH 9	−22.1			
+*erythro*-3-hydroxyaspartate				
+50 mM glutarate, pH 8.3	−35.23		~170	

[a] External standard: trifluoroacetic acid, 2 mM, pH 6.8. Positions given for benzene derivatives are the centers of multiplets arising from coupling of F to H atoms attached to ring. For pyridine, a broad singlet was observed in each case. [b] From Dewar & Kelemer (1968). [c] From Emsley & Phillips (1971), p 345.

8). However, there are two light absorption bands. These presumably represent the distinctly different tautomers **7** and **8**, which must be in rapid equilibration. Scott et al. (1984) showed that when the pH of E–FPLP is raised from 5.4 to 8 the −20.7 ppm band drops in intensity and broadens somewhat while a new resonance, representing the dipolar ion **12** arises at −36.2 ppm. Thus, despite the rapidity of its movement between the 3′-O and imine N, in the two tautomeric forms, the chelated proton in the internal Schiff base exchanges out slowly compared with the NMR time constant of ~0.2 ms.

The width of the −20.7 ppm resonance of E–6FPLP is strongly dependent upon the anion concentration, decreasing from 340 Hz in 10 mM cacodylate to 180 Hz upon addition of 0.3 M sodium chloride to ~1 mM enzyme (calculated as monomer). The half-saturating Cl^- concentration for inducing this narrowing is 10 mM at both pH 5.4 and pH 9. However, the width is also dependent on the protein concentration. The widths extrapolated to infinite dilution are ~230 and 130 Hz, respectively, for 10 mM cacodylate and 50 mM NaCl.

Although addition of as little as 10 mM 2-oxoglutarate raises the pK_a of E–FPLP to >9, it has no effect on the ^{19}F chemical shift. However, other dicarboxylates studied shift the −20.7 ppm resonance to more negative values (Figures 7B and 8) and at the same time increases the tautomeric ratio R_{sb}. The effect is greatest for L-malate and *meso*-tartrate and is very small for the 5-carbon glutarate. From Figure 7B it is apparent that in a plot of ^{19}F chemical shift vs. R_{sb} the sharp ^{19}F resonance of the 2-methylaspartate complex also lies close to the curve for simple dicarboxylate complexes. Titration with DL-2-methylaspartate shows that half-saturation is achieved

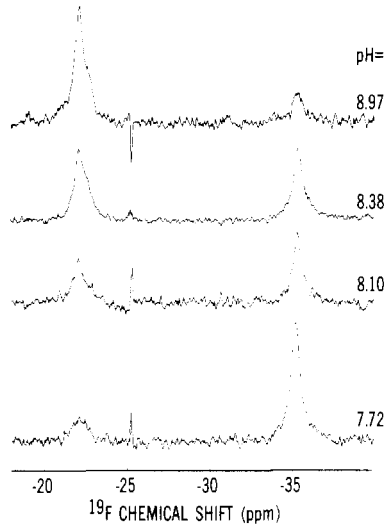


FIGURE 9: ^{19}F NMR titration of 6-FPMP-containing enzyme. The resonance at −36.4 ppm decreases and rises at −22.2 ppm with an increase in pH. The 6-FPMP was generated on the enzyme by treatment of the 6-FPMP-containing enzyme with cysteine sulfinate followed by gel filtration through a column of Sephadex G-25.

at ~2.2 mM in the L-amino acid component at pH 5.4. Even at saturation about ~24% of the ^{19}F continues to give a sharp resonance at −20.3 ppm (Figure 8) close to the position for free E–FPLP in addition to the major peak at −30.1 ppm when the temperature is 5 °C. However, this resonance broadens and decreases to ~10% of the total as the temperature is raised to 25 °C.

The ^{19}F NMR spectrum of 6-FPMP bound to the apoenzyme (E–FPMP) is a singlet at −35.3 ppm, which decreases and reappears at −22.1 ppm as the pH is raised from 5 to 9 around the spectrophotometrically determined pK_a of 8.2 (Figure 9). Thus, in this case too, the rate of exchange of the dissociable proton is slow on the NMR time scale. At pH 8.3 both the −22.1 and −35.3 ppm resonances are observed. When 50 mM sodium glutarate was added to such a solution, the −22.1 ppm peak almost disappeared and the −35.3 peak increased. This indicates that dicarboxylates (and presumably also monoanions) bind to the lower pH form of E–FPMP and raise its pK_a just as they bind to the low-pH form of E–PLP or E–FPLP.

The ^{19}F NMR spectra of complexes of E–FPLP with L-glutamate, L-aspartate, and L-*erythro*-3-hydroxyaspartate have been reported by Scott et al. (1984) and are summarized in Table IV. That of the glutamate complex is dominated by a broad resonance at −32 ppm but also always contains a smaller resonance at −20.5 ppm. At pH 8, 6 mM L-glutamate, these are the only resonances seen. However, with 1 mM glutamate the −20.5 resonance is larger and a small band is present at −32 ppm. These probably represent free E–FPLP together with some of the high-pH form of E–FPLP and E–FPMP, respectively, in an equilibrium mixture. Similarly, at pH 5.4, 50 mM glutamate, a distinct peak was present at −20.7 ppm, as well as a shoulder at −35.8 ppm. When sodium chloride was added up to a total of 300 mM, the major −32 ppm band almost disappeared as substrates were displaced from the enzyme. Both the −20.7 and −35.8 ppm bands of free enzyme increased correspondingly. At pH 9.1, 53 mM glutamate, the spectrum was similar to that at pH 5.6. A band for E–FPMP was present at −35.7 ppm and also a band at −22.1 ppm. This cannot be free E–FPLP at this pH but must be the high-pH form of E–FPMP. A small amount of free 6-FPMP also appears as a sharp resonance at −18.9 ppm.

At pH 7 in the presence of 1 mM L-aspartate the E–FPLP spectrum has a major fairly sharp resonance at −30.6 ppm, close to that for 2-methylaspartate, as well as a broader minor band at −21 ppm and a very weak E–FPMP band at −35 ppm. For E–FPLP plus 12 mM *erythro*-3-hydroxyaspartate, the spectrum is particularly sharp with a major band at −35.8 ppm, a minor band at −34.4 ppm, and often a sharp minor band at −18 ppm. Integration showed that at 2 °C the −35.8 ppm band contained 63% of the total area of bound coenzyme and the −34.4 ppm band 37%. The ratio changed from 1.7 at 2 °C to 2.0 at 20 °C.

In addition to the resonances from bound 6-FPLP, there was often a small sharp resonance between −43.2 and −44 ppm, which could be removed by dialysis. This was identified as free F^- whose resonance (for a 5×10^{-5}M solution) was −44.03 ppm. When NaF was added to a sample of E–FPLP that had a sharp peak at −43.76 ppm, a single peak at −43.79 ppm was observed. The shift in peak position of the F^- in the presence of the protein is probably a result of rapid exchange with fluoride bound to the protein. The release of F^- at 2 °C was very slow, the area of its peak amounting to only 0.5% of that of the bound coenzyme after 6 weeks at pH 5.4 in a 20 mM Tris–acetate buffer. However, at higher temperatures and in the presence of added substrates, the release was more rapid. No release of F^- was observed with free 6-FPLP or with the coenzyme bound to glycogen phosphorylase (Chang & Graves, 1985).

Discussion

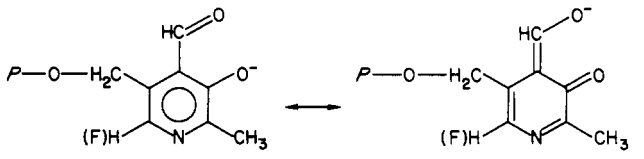
The positions of the light absorption bands of 6-fluoropyridoxine, 6-FPMP, and the 6-FPLP-containing Schiff base are shifted bathochromically by a small amount (3–12 nm) relative to the positions for the unfluorinated compounds (Table I of supplementary material). The shift is greater for anionic and cationic forms than for neutral molecules. The positions of the absorption band at 290 nm indicate conclusively that 6-FPMP in aqueous solutions exists below its pK of 7.45 with an uncharged ring. The same is true for 6-FPLP, which absorbs maximally at 356 nm below its pK of 7.8. These peak positions should be compared with those of PMP and PLP (327 and 391 nm, respectively) in their dipolar ionic ring forms. If the 6-fluoro compounds had dipolar ionic rings, they would be expected to absorb at even longer wavelengths. The pK values observed for the 6-fluoro coenzymes are clearly those of the phenolic group [see also Chang & Graves (1984)]. No significant effect of dissociation of the phosphate group, whose pK_a is about 6.2, is seen. Spectra of the three 6-halopyridoxines show that they too all exist as nondipolar ionic species with pK_a values for protonation of the ring nitrogen as low as −0.2. This is presumably a result of the strong inductive electron withdrawal by the halogen atom.

As is apparent from Table I, the electronic effect of a fluorine atom in this position is largely on the adjacent ring nitrogen. The microscopic pK_a of the cation drops from 5.6 in pyridoxine to ~0 in 6-fluoropyridoxine and from 3.6 in PLP to less than −1 in 6-FPLP. The effect of the fluorine on pK_a of the phenolic group, however, is insignificant, the pK_a values of 6-fluoropyridoxine, 6-FPMP, and 6-FPLP being almost identical with the corresponding microscopic pK_a values for pyridoxine, PMP, and PLP (Table I). These findings are predicted approximately by application of the Hammet equation (Hammett, 1970; Jaffe, 1955; Barlin & Perrin, 1966; Sanchez-Ruiz, 1983). The pK_a values of phenols are approximated by eq 3 (Barlin & Perrin, 1966). Here, the σ

$$pK_a = 9.92 - 2.23\sum\sigma \quad (3)$$

values are Hammett's substituent constants. Since the value of σ_p^- for −F (the appropriate constant for dissociation of phenols) is only 0.02, the expected pK_a of 6-FPLP is within 0.04 unit of the corresponding microscopic pK_a of PLP. The pK_a values of pyridines can be predicted approximately as $5.29 - 5.9\sum\sigma$ (Jaffe, 1955; Barlin & Perrin, 1966). Effects of ortho substituent are not easily predicted; thus, the apparent σ_o for −F is 0.93 for dissociation of benzoic acid but only 0.54 for dissociation of phenols. However, consider the microscopic pK_a of the pyridinium group in the cation of pyridoxine, which can be evaluated from published data (Metzler & Snell, 1955; Metzler et al., 1973) as 5.63. This is 5.8 units higher than that for 6-fluoropyridoxine and allows an estimation of σ_o for −F as 0.98, in agreement with that based on benzoic acids. Thus, the striking effect of the 6-fluoro substitution in lowering the pK_a of the *o*-pyridinium group drastically without affecting the pK of the *p*-phenolic group is in accord with the known electronic properties of fluorine on an aromatic ring.

The carbonyl group of PLP is highly hydrated at low pH, less so at neutral pH, and much less at high pH (Ahrens et al., 1970; Harris et al., 1976). This change in degree of hydration presumably reflects increasing donation of electrons from the phenolic oxygen into the carbonyl group (**9**), an



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interaction that should inhibit hydration. In its anionic form 6-FPLP is more hydrated than PLP, probably because the electron withdrawal by the fluorine (σ_m = 0.34) will counteract the electron donation by the phenolate ion. The unprotonated Schiff base of 6-FPLP with valine is also extensively hydrated to form the carbinolamine (Figure 6B).

Schiff bases of PLP in water exist largely as the ketoenamine **8**. For the Schiff base of PLP and valine, only about 7% can be the enolimine **7** (Metzler et al., 1980). For the Schiff base of 6-FPLP in water, as estimated from the areas of the 426- and 345-nm absorption bands, the ketoenamine still predominates but ~26% is enolimine. This change is qualitatively predicted from the electron withdrawal by fluorine (σ_m = 0.34). The resultant lowering of the basicity of the imine nitrogen allows the proton to shift to the phenolic oxygen.

For 6-H-containing Schiff bases the intensity of light absorption (as judged by the molar area) of tautomer **7** is about 60% of that of **8**. For the Schiff base of 6-FPLP plus valine, it is about 78% as great as for tautomer **8**. As is true for other related Schiff bases, the tautomeric ratio R = [**7**]/[**8**] increases as the polarity of the solvent drops. The sum of the areas of band 1 (**8**) + band 2 (**7**)/0.78 in water–methanol mixtures is nearly constant (supplementary material, Table II), an indication of the correctness of our analysis. The chemical shift of the single ^{19}F NMR resonance also changes considerably with solvent composition. When the ^{19}F chemical shift is plotted against the fraction of ketoenamine **8** from Table II of the supplementary material, a nearly linear relationship is observed (Figure 7A). The chemical shifts extrapolated from the curve in this figure are −14.7 and −21.8 ppm, respectively, for pure **7** and **8**. This change (+7.1 ppm) is just the same as that for protonation of the 4-fluorophenolate ion (Table III).

From the positions of the absorption maxima it appears certain that the 6-FPLP is present in the enzyme above the pK_a of 7.1 as a Schiff base with a dipolar ionic ring form. Thus, the bound FPLP absorbs maximally at 446 nm, close to the 431 nm of the PLP in the native enzyme. Similarly, the dipolar ionic form of 6-FPMP, with a maximum absorbance at 341 nm, is present in the enzyme below the pK_a of 8.2. In both instances the absorption bands are shifted 9–12 nm toward longer wavelengths compared to those of native enzyme in which PLP and PMP are believed to have dipolar ionic rings.

In view of the strong tendency for 6-FPLP and 6-FPMP to exist in nondipolar ionic forms, it may seem surprising that these substituted coenzymes bind well to apo-aspartate aminotransferase and have a high catalytic activity. This is easier to understand if we consider hypothetical microscopic pK_a values of groups in the coenzyme. In a Schiff base of PLP with an amine such as *n*-butylamine in water, the ring nitrogen has a microscopic pK of ~6.3 (Kallen et al., 1984) and dissociates before the protonated imine. The pK_a of the protonated imine of tautomer **8** then rises to >12. However, the active site of aspartate aminotransferase is designed to bind the dipolar ion. When the ring nitrogen is methylated, the pK of the imine with valine is lowered to about 9.6 in H_2O (Chen, 1981). N-Protonation should have a similar effect. The hydrogen bond from Tyr-225, together with the nearby positive charge of Arg-386 in the active site of the enzyme (Figure 1), evidently acts to lower the pK_a still further to about 6.4. We can also expect that the pK_a of the proton on the ring nitrogen will be *raised* perhaps by 3 units or more to above 9 when the imine nitrogen is unprotonated. As a consequence, the ring nitrogen remains fully protonated in the native enzyme as an ion pair with the carboxylate of Asp-222. The pK_a of the latter, by virtue of its hydrogen bonding to His-143, may be considerably lower than 5. These are hypothetical pK_a values and, in fact, the proton is chelated and held tightly in the ion pair. No change in absorption spectrum indicative of actual dissociation is observed up to pH 10 or above. It is a little hard to estimate how much change would be expected for such a dissociation, however. The dipolar ionic ring form of the Schiff base of *N*-methyl-PLP with valine absorbs at 360 nm whereas the anionic form of the corresponding Schiff base with PLP itself absorbs at 349 nm. From Figure 2B it seems almost certain that the anionic Schiff base of 6-FPLP, which exists in an equilibrium mixture with an equal amount of carbinolamine, does not absorb at as high a wavelength as the 375 nm of the enzyme-bound 6-FPLP.

The pK_a for the ring nitrogen in 6-fluoropyridoxine is about −0.2, and that of 6-FPLP is at least as low as −1, 4.6 units below the microscopic pK_a of 3.6 for the ring nitrogen of PLP. If the pK_a for the pyridinium nitrogen of a Schiff base of 6-FPLP in the enzyme is decreased from the greater than 9 estimated for a Schiff base of PLP in the active site by 4.6 units, its pK_a might be less than 5. However, the fact that the proton remains on the ring nitrogen rather than shifting onto the carboxylate of Asp-222 suggests that the pK_a of the ring nitrogen may not be this low. Perhaps in the relatively low dielectric surroundings of the active site the interaction between the phenolate ion and the ring nitrogen is strengthened, raising the pK_a of the latter. On the other hand, the pK_a of the Asp-222 carboxyl could also be quite low, especially if the cluster of buried imidazole groups of His-143, His-189, and His-193 is protonated. It is the difference in pK_a between the ring nitrogen of the coenzyme and the Asp-222 carboxylate that will determine where the proton is located.

Similar considerations apply to bound 6-FPMP. The microscopic constant for the ring nitrogen when the phenolic

group is deprotonated (but with $-CH_2NH_3^+$ present) might be about 4.6 units below the value of 8.5 observed for PMP. However, the ring nitrogen does remain protonated in the enzyme at pH 5. This again suggests that the pK_a of Asp-222 may be unusually low. The appearance of a pK of 8.2 with a shift of the absorption band to lower wavelengths suggests strongly that the ring nitrogen becomes deprotonated at high pH. This is in agreement with the conclusion that the microscopic pK_a of the protonated ring nitrogen should be lower for bound 6-FPMP than for bound 6-FPLP. The fact that E–FPLP does not display the pK_a may also reflect the presence of the Schiff base linkage, which may help to keep the protein tightly folded around the coenzyme. For 6-FPMP, we conclude that the binding of this coenzyme as an ion pair with Asp-222 has increased the apparent pK_a of its ring nitrogen from our estimate of ~4 to 8.2.

For the native enzyme the results of X-ray crystallography (Arnone et al., 1985a; Ford et al., 1980; Jansonius et al., 1985) and of resonance Raman spectroscopy (Benecky et al., 1985; Kallen et al., 1985) make it clear that in the low-pH ketoenamine form the pyridine ring in the aspartate aminotransferases is protonated on the ring nitrogen atom. However, for E–FPLP at low pH the state of protonation of the coenzyme ring is less certain. X-ray diffraction measurements by Arnone et al. show that in the crystalline enzyme 6-FPLP is bound in the same place and with the same orientation as is PLP in the native enzyme. However, protonation of the $-O^-$ group to give the predominant enolimine tautomer will decrease the pK_a of the ring nitrogen from its already low value by as much as 3 units or more. This could easily cause the proton to move from the ring nitrogen onto the carboxylate of Asp-222 and greatly weaken the binding of the coenzyme.

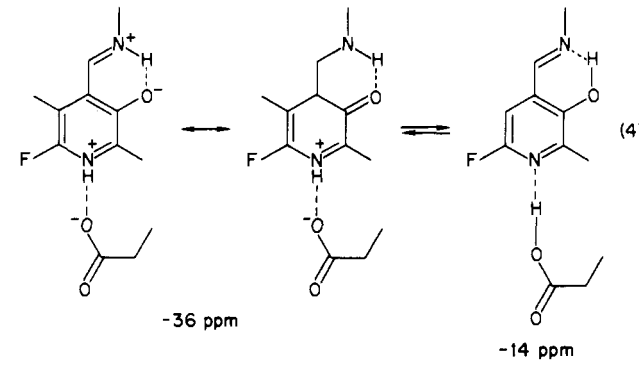
Observation of the ^{19}F chemical shifts provides another means of monitoring the state of protonation of the ring. Fluorine chemical shifts are primarily responsive to inductive effects of substituents in a ring. They can be predicted semiquantitatively from the shifts for monosubstituted fluorobenzenes (Gutowsky et al., 1952), which are almost additives. Thus, with data from Gutowsky et al. and from Table IV the predicted chemical shift for 5-deoxy-6-FPMP is −38.2 (fluorobenzene) −0.9 (2-CH_3) −10.6 or 11.3 (3-OH) + 1.0 (4-$CH_2NH_3^+$) −5 (5-CH_3) + 42.95 (ring N) = −10.7 to −11.5 ppm. Considering the magnitude of the individual shifts and the fact that the 5-$CH_2OPO_3H^-$ group is significantly less electron donating than CH_3, this is satisfactorily close to the observed value of −8.2 ppm for 6-FPMP at low pH.

Data on ^{19}F chemical shifts when an adjacent ring nitrogen is protonated are not readily available from the literature. The titration curve of 6-fluoropyridoxine in Figure 6A suggests that this shift amounts to as much as 14 ppm. Protonation of the ring nitrogen always leads to shifts to higher frequency for an adjacent 6-H nucleus. The large opposite shift for the 6-F nucleus is surprising. However, we believe that protonation does occur on the ring nitrogen. The positions of the ultraviolet absorption bands of the N-protonated 6-fluoropyridoxine are consistent with this conclusion. Thus, the position of the absorption maximum of the cation of 6-fluoropyridoxine is only 11 nm higher than that of the cation of pyridoxine, about the expected amount. The heights and shapes of the two bands are also similar.

What changes in chemical shift will accompany binding to a protein? Solvent effects of a few parts per million are possible. Since the fluorine atom in the enzyme lies almost in contact with the ring of Trp-140, ring current shifts are also possible. These should be relatively small for ^{19}F but must be considered. However, as a first approach we will ignore these effects and ask how much can be understood in terms of simple acid–base chemistry.

The chemical shift of bound 6-FPMP at high pH is −22.1 ppm, only 3.2 ppm more negative than for the free coenzyme with anionic ring and protonated amino group. Likewise, at low pH the −35.3 ppm chemical shift is only ~3 ppm more negative than that predicted for coenzyme with a dipolar ionic ring and protonated amino group. This is −8.2 for free 6-FPMP monocation with a neutral ring (Table IV) − 14 (protonation of ring N) − 10 (dissociation of phenolic hydroxyl) = −32.2 ppm. Bound 6-FPLP above its pK_a has an ^{19}F chemical shift of −36.2 ppm, 5.4 ppm more negative than is predicted by adding −14 to the chemical shift for the anionic ring form of the Schiff base with valine (Table IV). Although there is an additional shift of −3.2 to −5.4 ppm for binding to the protein, these results appear to confirm the conclusion based on the electronic spectra that bound 6-FPMP has a dipolar ionic ring below its pK_a and that the enzyme-bound Schiff base of 6-FPLP exists as a dipolar ion above its pK_a.

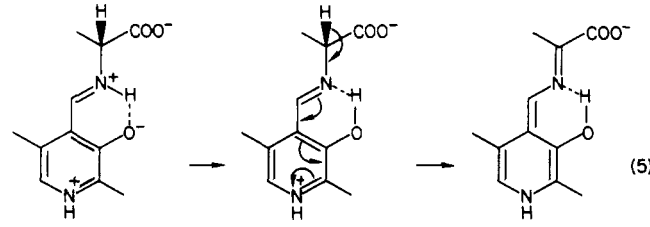
The most surprising conclusion from the ^{19}F NMR measurements is that at low pH there is rapid equilibration between the enolimine and ketoenamine tautomers and that the former is not protonated on the ring nitrogen but that the latter is (eq 4).



(4)

This conclusion follows directly from Figure 7B, which shows the fraction of ketoenamine for various dicarboxylate complexes as well as free enzyme plotted vs. ^{19}F chemical shift. The lower value, for pure enolimine, is about −13.4 ppm, quite close to the limit of −14.7 ppm for the model Schiff base (Figure 7A). However, the upper limit of the chemical shift, for pure enzyme-bound ketoenamine is about −34.5 ppm, 12.7 ppm more negative than for the model Schiff base and strongly suggesting N-protonation of the ring.

Because the basicity of the ring nitrogen is so low in 6-FPLP, the enzyme reconstituted with 6-FPLP would not seem to be a very close model for the native enzyme. However, it does have 40% as much catalytic activity. It is possible that during catalysis by the enzyme deprotonation of the ring nitrogen is required at some stages as was suggested by Ivanov & Karpeisky (1969) and more recently by others (Metzler, 1979; Arnone et al., 1984b). It is also possible that enolimine formation precedes loss of the α proton of a substrate in a rate-limiting step of enzymatic catalysis (eq 5). Having this



(5)

tautomerization step on the reaction pathway would prevent the phenolate oxygen from interfering with the removal of the α proton by donation of electrons into the ring (Matsushima & Matsumoto, 1974; Ledbetter et al., 1981; Kallen et al., 1985). This suggests the further possibility that the tautomerization to the enolimine could occur as in eq 4 and that the loss of the α-H (second step of eq 5) could occur by a synchronous transfer of the proton back from Asp-222 onto the ring as the α-H was removed. This would have the advantage of not requiring participation of any "buried" isolated electrically charged groups. Perhaps a more likely possibility, however, is that the proton in native enzyme is always on the ring nitrogen but that when the ketoenamine tautomer is present the H-bond to Asp-222 is weak and is replaced by an H-bonded ion-pair interaction with a protonated His-143 side chain while when enolimine is present the H-bond to Asp-222 is strong (Figure 1).

We have interpreted our observations to mean that 6-FPLP provides a sensitive indicator of the state of protonation of the pyridine ring. Alterations in this state of protonation may, in turn, reveal alterations in the strength of the hydrogen bond between the ring nitrogen and Asp-222. For example, we have interpreted the changes in ^{19}F chemical shift and the correlated changes in tautomeric ratio R_{sb} as indicating that the binding of L-malate and of *meso*-tartrate leads to more N-protonation of the 6-FPLP than does binding of succinate or acetylenedicarboxylate. This may mean that there is a tighter interaction between the α-carboxylate group and the guanidinium group of Arg-386 with malate and *meso*-tartrate than with the other two dicarboxylates. When this interaction is strengthened, that between Arg-386 and the 3-O^- group of the coenzyme is weakened, allowing electrons to enter the ring and to hold the proton more tightly to the ring nitrogen. In the case of 6-FPLP, this leads to a greater proportion of ketoenamine tautomer. It is not as clear how this may affect the strength of the hydrogen bond. Electron donation into the ring will neutralize the positive charge and weaken the electrostatic interaction with the Asp-222 anion. With 6-FPLP, however, in the absence of the electron donation from the 3-O^-, the proton will shift from the ring N to the aspartate carboxylate, thereby destroying the ion pair and presumably leaving a weaker hydrogen bond. In the native enzyme the ion pair will always be maintained, but the hydrogen bond will be weakened when donation of electrons into the ring from the 3-O^- occurs.

It is not at all clear why binding of 2-oxoglutarate in its nonproductive complex with the aldimine form of the enzyme or of glutarate with the same form has little or no effect on the tautomeric ratio or ^{19}F chemical shift. Perhaps the initial binding does not perturb the coenzyme, but following a conformational change, which does not occur as readily with the 5-carbon dicarboxylates, the interaction between the incoming carboxylate group and the coenzyme is enhanced.

Our NMR data indicate that the proton is probably bound to the ring nitrogen most tightly in the "external aldimine" of the 2-methylaspartate complex. From the electronic spectra we estimated the tautomeric ratio R_{sb} as 0.46 (Figure 7B). However, because of the complexity of the absorption spectrum (Figure 5), this value is uncertain. From the ^{19}F chemical shift it would have to be $\sim$0.25 to lie on the least-squares line in Figure 7B. In any case, if our interpretation is correct, the hydrogen-bonded ionic interaction between the Asp-222 carboxylate and protonated ring nitrogen will be weaker in the methylaspartate complex than in the free enzyme at low pH or in other dicarboxylate complexes. This may be mechanistically significant in allowing for easy tilting over of the coenzyme ring and in providing a powerful "electron sink" to assist in removal of the α-hydrogen atom of a substrate.

The binding of monoanions to E–FPLP appears to be similar to that with native enzyme. The dissociation constant of 10 mM for the chloride complex estimated from line broadening is identical with that estimated in the same way from the ^{19}F NMR resonance for the enzyme labeled with a trifluoromethyl-containing group on cysteine-390 at pH 8.2 (Critz & Martinez-Carrion, 1977) or from binding of perfluorosuccinate at pH 7 (Martinez-Carrion et al., 1973). It is also the same as the dissociation constant for chloride estimated spectrophotometrically by Chen et al. (1971) from competition with *erythro*-3-hydroxyaspartate. It is quite close to that of 21 mM estimated spectrophotometrically for native enzyme at low pH (Jenkins, 1980). However, from the spectrophotometrically determined increase of 1.0 unit in the pK_a of the enzyme when the Cl^- complex is formed, it follows that the dissociation of the chloride ion from the α-carboxylate binding site (adjacent to the coenzyme imine group) must be 0.21 M at high pH (Jenkins, 1980). The explanation for the difference between this value and the 10 mM determined by NMR spectroscopy and competitive binding with *erythro*-3-hydroxyaspartate may be that the latter measures the binding of chloride to the guanidinium group of arginine-292, the binding site for the side-chain carboxylate of substrates. Alternatively, chloride ions may decrease the interaction between the protein molecules. This interaction is presumably the cause of the band broadening seen at high protein concentrations.

Especially narrow band widths are observed for the 2-methylaspartate complex (90 Hz) and the *erythro*-3-hydroxyaspartate complex (100 Hz). Critz & Martinez-Carrion (1977) also observed a pronounced narrowing from $\sim$100 to 55 Hz upon binding of glutarate to the Cys-390-labeled enzyme. They attributed this to an increase in segmental notion of the Cys-390 peptide loop in the complex. A similar explanation seems unlikely in our case, however. The coenzyme ring bearing the fluorine atom is probably not moving rapidly in the slowly reacting *erythro*-3-hydroxyaspartate complex and may also be relatively immobile in the 2-methylaspartate complex. We are investigating effects of field strength, dilution, and buffer anions on band width. While we are not yet prepared to offer a complete explanation, the data suggest that in the 2-methylaspartate complex the coenzyme is relatively immobile and that the broader signals in other complexes result from chemical shift anisotropy or from rapid interconversions between different complexes.

Another puzzle concerns the presence of the −20.3 ppm resonance in the 2-methylaspartate complex. At low temperature it accounts for about 24% of the total ^{19}F signal. Perhaps it represents a Michaelis complex that is in slow equilibrium with the external aldimine or a nonproductive complex of D-2-methylaspartate. The chemical shift is close to that of the free enzyme, but it is difficult to understand this or to relate it to the observed absorption spectrum.

From the electronic spectra the glutamate complex can be estimated to contain as much as 82% of PMP-containing species (ketimine, carbinolamine, Michaelis complex), which give rise to the 332-nm band. In addition, about 7% absorbs at 378 nm and 10% at 454 (ketoenamine) while only 1% is the quinonoid intermediate. The single broad resonance at −32 ppm is consistent with the presence of a rapidly equilibrating mixture of these species. The L-aspartate complex, like that of 2-methylaspartate, also contains a minor band at −21 ppm but the major band at −30.6 ppm is both much sharper and displaced to a higher frequency than that of the

glutamate complex. This suggests a different distribution of species and perhaps a different rate of interconversion than that with glutamate. On the other hand, the complex with *erythro*-3-hydroxyaspartate contains two sharp resonances. Comparison with the electronic spectrum and the assumption of a molar absorptivity of 41 000 M^{-1} cm^{-1} for the quinonoid form (Kallen et al., 1985) indicate that the −35.8 ppm band must be the quinonoid species. Integration of the NMR spectrum indicate 63% quinonoid form. The second band, at −34.4 ppm, probably corresponds to the 390-nm band in the electronic spectrum, the 330-nm band probably representing a second transition for the quinonoid intermediate. On this basis, the 390-nm band could represent an initial Michaelis complex with both an absorption spectrum and NMR resonance consistent with a dipolar ionic structure (Figure 1).

The introduction of the fluorine atom into PLP makes possible a simple spectrophotometric approach to measurement of the binding of anions to the 6-FPMP form of the enzyme. This is to observe the shift in the pK_a of 8.26 caused by anion binding, the same approach used by Jenkins and associates [reviewed by Jenkins & Fonda (1985)] for the native enzyme in the PLP form.

Cytosolic aspartate aminotransferase catalyzes the transamination reaction at a rate of one turnover in about 5 ms (Kiick & Cook, 1983). The time constant for exchange of the imine proton of the 6-FPLP-containing enzyme estimated with the peak separation (Hull & Sykes, 1975) from NMR titrations (Scott et al., 1985) is ≥0.2 ms per exchange. This would allow ≤25 proton exchange events during one catalytic cycle. Similar considerations apply to the proton on the ring nitrogen of F-PMP (Figure 8).

A slow generation of F^- from bound 6-FPLP occurred at higher temperatures and in the presence of substrates. A mechanism involving protonation on C-6 by electron donation from the phenolate oxygen followed by elimination of F^- and concomitant loss of a proton at C-4′ from a carbinolamine, ketimine, or 6-FPMP form is possible. If this occurred from bound 6-FPMP, the product would be enzyme-bound PLP. However, this is a very slow process, and in the 5-min assays used in obtaining the data of Table III no significant conversion of 6-FPLP to PLP could have occurred.

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SUPPLEMENTARY MATERIAL AVAILABLE

Three tables showing peak positions and areas for lognormal curves used to resolve spectra, fractions of various species present, and molar areas assumed or measured for these species (5 pages). Ordering information is given on any current masthead page.

Registry No. 6-FPMP, 42242-44-4; PMP, 529-96-4; 6-FPLP, 90932-80-2; PLP, 54-47-7; AspAT, 9000-97-9; *p*-HOC_6H_4F, 371-41-5; F^-, 16984-48-8; PhF, 462-06-6; *m*-$H_2NCH_2C_6H_4F$, 100-82-3; *meso*-$HO_2C(CHOH)_2CO_2H$, 147-73-9; $HO_2CC{\equiv}CCO_2H$, 142-45-0; 6-fluoropyridoxine, 42242-41-1; 6-chloropyridoxine, 15741-67-0; 6-bromopyridoxine, 50441-54-8; pyridoxine, 65-23-6; Schiff base with DL-valine and 6-FPLP, 99098-28-9; 2-fluoropyridine, 372-48-5; 2-methylaspartic acid, 866-73-9; L-glutamic acid, 56-86-0; DL-*erythro*-3-hydroxyaspartic acid, 6532-76-9; succinic acid, 110-15-6; glutaric acid, 110-94-1; 2-oxoglutaric acid, 328-50-7; aspartic acid, 56-84-8.

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Reversible Blocking of Half-Cystine Residues of Proteins and an Irreversible Specific Deamidation of Asparagine-67 of *S*-Sulforibonuclease under Mild Conditions†

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ABSTRACT: For use in protein-folding studies, a rapid procedure for the preparation of octa-*S*-sulforibonuclease A (SO_3-RNase A) with 2-nitro-5-(sulfothio)benzoate is described. The modification is specific for thiols and disulfide bonds. The modified protein was characterized and found to be enzymatically inactive and predominantly conformationally disordered. In the absence of thiols, the modified sulfhydryl groups were found to be stable over the pH range of 2–9. However, when the modified protein is incubated at neutral to slightly alkaline conditions for prolonged periods of time or at elevated temperatures, it undergoes a further (irreversible) modification that decreases its net charge at pH 8.0. Evidence is presented that demonstrates that this additional modification is due to the specific deamidation of asparagine-67. When incubated with an excess of reduced and oxidized glutathiones for 24 h at pH 8.2 and 25 °C, the reversible sulfo blocking group was removed, and essentially quantitative (94%) native enzymatic activity was regenerated from both SO_3-RNase A and its deamidated derivative (SO_3-RNase B). Although the two fully active refolded species differ in their elution behavior on ion-exchange chromatography, they are indistinguishable by many other methods. The significance of this finding for studies of the folding of RNase A is discussed.

There is much current activity using recombinant DNA techniques to produce various proteins. If the protein of interest contains disulfide bonds, it is necessary to pair the half-cystine residues properly to produce the native structure. While such a regeneration process is, in principle, a spontaneous one (Anfinsen, 1973), it is often hampered by practical difficulties, such as insolubility of the reduced material, and various methods have been proposed to fold the protein properly (Hayenga et al., 1983). However, such procedures are often cumbersome and, sometimes, are difficult to implement. It is, therefore, worthwhile to develop a suitable alternative procedure to obtain reversibly modified, soluble forms of the reduced protein that are chemically homogeneous,

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