Porcine Cytosolic Aspartate Aminotransferase Reconstituted with [4′-^{13}C]Pyridoxal Phosphate. pH- and Ligand-Induced Changes of the Coenzyme Observed by ^{13}C NMR Spectroscopy†

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ABSTRACT: Apoenzyme samples of aspartate aminotransferase (AspAT) purified from the cytosolic fraction of pig heart were reconstituted with [4′-^{13}C]pyridoxal 5′-phosphate (pyridoxal-P). The ^{13}C NMR spectra of AspAT samples thus generated established the chemical shift of 165.3 ppm for C4′ of the coenzyme bound as an internal aldimine with lysine 258 of the enzyme at pH 5. In the absence of ligands the chemical shift of C4′ was shown to be pH dependent, shifting 5 ppm upfield to a constant value of 160.2 ppm above pH 8, the resulting pK_a of 6.3 in agreement with spectrophotometric titrations. The addition of the competitive inhibitor succinate to the internal aldimine raises the pK_a of the imine to 7.8, consistent with the theory of charge neutralization in the active site. In the presence of saturating concentrations of 2-methylaspartic acid the C4′ signal of the coenzyme was shown to be invariant with pH and located at 162.7 ppm, midway between the observed chemical shifts of the protonated and unprotonated forms of the internal aldimine. The intermediate chemical shift of the external aldimine complex is thought to reflect the observation of an equilibrium mixture composed of roughly equal populations of the protonated ketoenamine and a dipolar anion species, corresponding to their respective spectral bands at 430 and 360–370 nm. Conversion to the pyridoxamine form was accomplished via reaction of the internal aldimine with L-cysteinesulfinate or by reduction with sodium borohydride, and the resulting C4′ chemical shifts were identified by difference spectroscopy. Finally, the line widths of the C4′ resonance under the various conditions were measured and qualitatively compared. The results are discussed in terms of the current mechanism and molecular models of the active site of AspAT.

Aspartate aminotransferase (E.C. 2.6.1.1) is the most extensively studied member of the pyridoxal-requiring enzymes [see reviews of Braunstein (1973), Christen and Metzler (1985), and Jansonius and Vincent (1987)]. The minimal mechanism of AspAT,[1] as deduced by the enormous wealth of model, spectroscopic, and X-ray crystallographic studies, entails the reversible transamination of α-amino acid and α-keto acid substrates catalyzed by the interconversion of the bound coenzyme between its pyridoxal and pyridoxamine forms (Scheme I). Proton NMR spectroscopy has been used quite extensively for structural studies of various pyridoxal compounds and has provided a variety of information on the chemical mechanism of pyridoxal catalysis. The application of this technique to the study of the macromolecular pyridoxal enzymes, however, is hampered by the complexity of their spectra due to the large number of protein-associated protons confined in a narrow chemical shift range and by the broadening of individual resonances via homonuclear coupling and the inherent increase in line widths characteristic of large proteins. In contrast, proton-decoupled ^{13}C NMR has a wider chemical shift range and is devoid of homonuclear and heteronuclear couplings; hence, the simplicity of the spectra promised potential usefulness for the study of pyridoxal-requiring enzymes. Particularly, ^{13}C enrichment at a given position in a macromolecule or its ligand enables acquisition of valuable information on the interaction between a macromolecule and its ligand through the chemical shift behavior of the incorporated ^{13}C nucleus (Mackenzie et al., 1984). On the basis of the minimal mechanism presented in Scheme I, it is expected that C4′ of the coenzyme (●), which has been shown to form the internal aldimine bond with Lys 258 of the enzyme, would serve as a sensitive chemical shift probe for the ionization state of the imine nitrogen and its microenvironment. Accordingly, the present paper reports on the ^{13}C NMR spectroscopy of the porcine cytosolic isoenzyme of AspAT reconstituted with [4′-^{13}C]pyridoxal-P. The pH-dependent chemical shift changes at C4′ of the coenzyme in the enzyme alone and its external aldimine complex with 2-methylaspartate and the effect of other ligands are described, together with structural and mechanistic questions that these data pose.

Experimental Procedures

Materials. Aspartate aminotransferase (AspAT) exists in two isozymic forms in mammalian tissues: one localized in the cytosol and the other in mitochondria. Cytosolic AspAT

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[1] Abbreviations: AspAT, aspartate aminotransferase; pyridoxal-P, pyridoxal 5′-phosphate; pyridoxamine-P, pyridoxamine 5′-phosphate; NMR, nuclear magnetic resonance; FID, free induction decay; TSP, 3-(trimethylsilyl)propionate; KPB, potassium phosphate buffer; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetate; MeAsp, 2-methyl-DL-aspartic acid.

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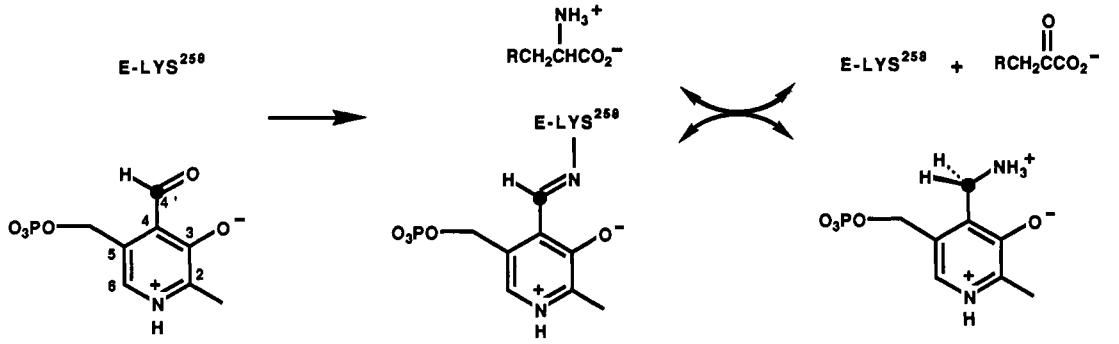
Scheme I



is present in three subforms (α, β, and γ), the α subform being the most active and used in these experiments. The α subform of the cytosolic AspAT was purified from pig heart by the method of Jenkins et al. (1959) with modifications as previously described (Martinez-Carrion et al., 1967; Morino et al., 1977). Its spectral and catalytic properties were as follows: A_{430nm}/A_{340nm}(pH 5) = 3.6–3.7; specific activity = 290 μmol min^{-1} mg^{-1} at 25 °C. The enzyme activity was determined by measuring the absorbance at 280 nm for the formation of oxalacetate in the reaction mixture containing 20 mM L-aspartate and 10 mM 2-oxoglutarate in 1.0 mL of 0.1 M Tris-HCl buffer (pH 8.0). 2-Methyl-DL-aspartate, cysteinesulfinic acid, and sodium borohydride were obtained from Sigma.

Preparation of AspAT Apoenzyme and Reconstitution with [4′-^{13}C]Pyridoxal-P. The pyridoxal form of AspAT (200 mg, 4.3 μmol as monomeric unit) in 8 mL of 0.1 M potassium phosphate buffer (KPB) (pH 8.0) was converted into the pyridoxamine-P form by reacting with 1 mM cysteinesulfinate. The pyridoxamine-P was then dissociated in 1 M KPB (pH 5), and the apoenzyme was isolated by gel filtration chromatography using a Sephadex G-50 column (2.8 × 50 cm). The apoenzyme fraction was concentrated and dialyzed against 20 mM KPB (pH 6.0) containing 0.1 mM EDTA and 0.01 mM dithiothreitol. The resulting apoenzyme was incubated for 30 min at room temperature with an 1.2-fold molar excess of [4′-^{13}C]pyridoxal-P (99 ^{13}C atom %) prepared as described previously (Van Velde et al., 1985). The holoenzyme was then separated from the remaining free pyridoxal-P by size-exclusion membrane filtration (Amicon YM-10 membrane). The typical range of reconstitution was 94–96%. The reconstituted enzyme was maintained as the pyridoxal form (~50 mg/mL) at 4 °C in 10 mM KPB (pH 6) in the presence of 50 mM succinate to stabilize the enzyme. This procedure for preparation of the pyridoxamine form and the apoenzyme is based on that described previously (Jenkins & D'Ari, 1966a; Scardi et al., 1963; Morino et al., 1986).

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were recorded on either Bruker WM-300 wide bore or AM-500 spectrometers equipped with Aspect 2000 computers. With the exception of the pH titrations, which were recorded at 75.46 MHz in a 10-mm tube, all measurements were recorded at 125.76 MHz in a 5-mm tube. Internal deuterium oxide (10–20%) was employed as the lock solvent. In general, the NMR samples were prepared by replacing stock solutions of AspAT (previously dialyzed against 10 mM KPB to remove succinate if necessary) with 10 mM KPB/D_2O buffer by repeated dilution and concentration in a 3-mL Amicon stirred cell (YM-10 membrane) to final protein concentrations of 100–200 mg/mL (1–2 mM in dimer). The pH of the NMR sample was adjusted by either 1 M NaOH or 1 M H_3PO_4 and was uncorrected for the effect of deuterium on pH. The pK_a values

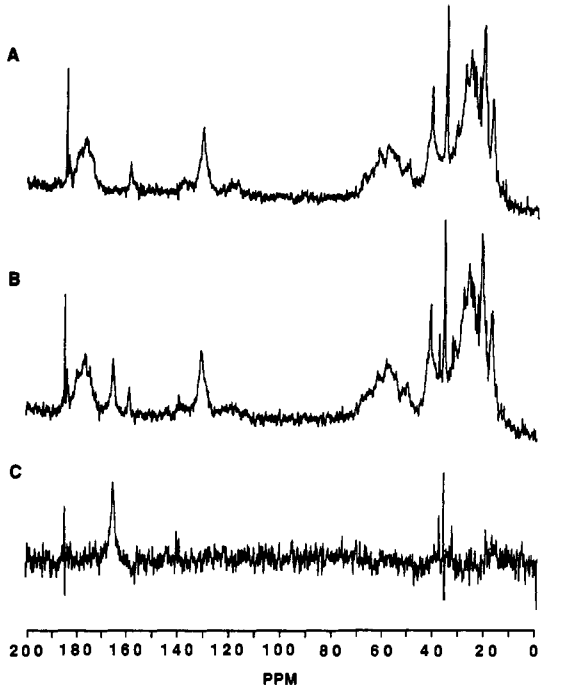


FIGURE 1: Proton-decoupled 125.76-MHz ^{13}C NMR spectra of (A) AspAT isolated from cytosolic pig heart and (B) apoenzyme sample of AspAT reconstituted with [4-^{13}C]pyridoxal-P; (C) difference spectrum (B − A). Conditions: 2.0 mM AspAT in 20 mM KPB (pH 6.0) containing 50 mM succinate. Number of scans (A) 70 000 and (B) 60 000.

were obtained by fitting the pH titration data to the Hill equation by nonlinear least-squares calculations (Markley, 1973). Low-power proton decoupling was accomplished by using the WALTZ sequence available in the Bruker software. Approximately 60° pulse widths were employed with acquisition and recycle times of 0.31 and 0.5 s, respectively, for 125 MHz data and 0.5 and 0.3 s, respectively, for 75 MHz data. In most cases 20 Hz exponential line broadening was applied to the 16K data point FID prior to Fourier transformation. Chemical shift values were reference to external TSP contained in a coaxial tube.

RESULTS

^{13}C NMR Spectra of AspAT Reconstituted with [4′-^{13}C]-Pyridoxal-P. Cytosolic AspAT from porcine heart (M_r = 92 688) is composed of two identical subunits of 412 amino acid residues. The natural abundance ^{13}C NMR spectra of native AspAT is shown in Figure 1A. Typical for a protein of high molecular weight, the spectrum of AspAT can be characterized into three groups of poorly resolved resonance lines: aliphatic carbons (10–70 ppm), aromatic carbons (120–135 ppm), and carbonyl carbons (170–180 ppm). In addition, a small signal at 160 ppm is observed, which ori-

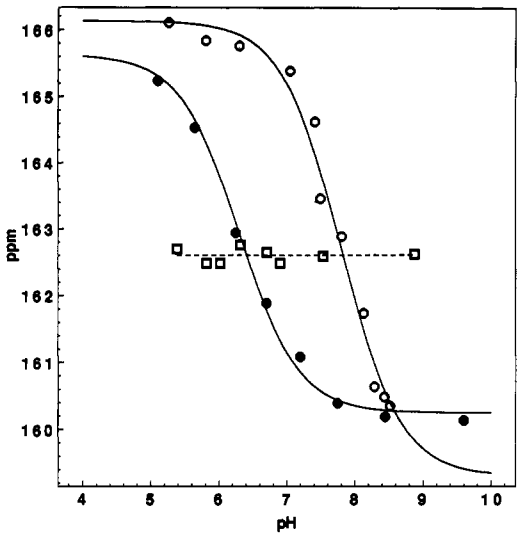


FIGURE 2: pH dependence of the coenzyme C4′ resonance of the pyridoxal form of [4′-^{13}C]pyridoxal-P reconstituted AspAT in the absence of ligands (●) and in the presence of 10 mM succinate (○) or 50 mM 2-methyl-DL-aspartate (□). In the case of unliganded coenzyme and in the presence of succinate, the lines represent the least-squares fit of the data producing the following pK_a (Hill coefficients in parentheses): unliganded, 6.29 ± 0.02 (0.79); succinate, 7.81 ± 0.02 (1.03).

ginates from the ζ-carbons of arginine and tyrosine residues. Finally, the two sharp signals at 184 and 36 ppm represent respectively carboxyl and methylene carbons of succinate, which was included in the sample to stabilize the enzyme. A sample of AspAT apoenzyme was prepared and incubated with a stoichiometric amount of [4′-^{13}C]pyridoxal-P as described under Experimental Procedures. The ^{13}C NMR spectrum of the reconstituted AspAT obtained under similar conditions revealed a new resonance line at 165.9 ppm (Figure 1B). The difference spectrum (Figure 1C) obtained by subtracting the unlabeled enzyme spectrum from the ^{13}C-labeled spectrum clearly demonstrates the complete cancellation of all signals originating from the protein with the exception of one remaining signal that can be attributed to ^{13}C enrichment at C4′ of the bound pyridoxal-P.

Effect of pH. The ionization behavior of the internal aldimine in the absence of ligands was investigated by utilizing the ^{13}C-enriched pyridoxal-P. The reconstituted enzyme was freed of succinate via dialysis against KPB (pH 4.8) and again examined by ^{13}C NMR. At this pH, the chemical shift of C4′ of the internal aldimine was determined to be 165.3 ppm with a line width of 125 ± 10 Hz. The effect of chemical shift versus pH is shown in Figures 2 and 3A,D. As represented by curve (●), the chemical shift for C4′ of the bound cofactor was found to undergo a 5 ppm shift to higher field with increasing pH, with a pK_a value of 6.3. This value is in good agreement with that determined spectrophotometrically for the dissociation of the proton from the coenzyme aldimine nitrogen[2] (Jenkins et al., 1959). Thus, C4′ senses the ionization state of the imino group. This is in contrast to results of previous proton NMR experiments in which the coenzyme 4′-proton was shown to be insensitive to the ionization state

[2] The pK_a value is dependent upon the ionic strength and falls to about 5.25 when extrapolated to zero ionic strength (Bergami et al., 1968). Under the present condition ($\mu > 0.07$), the pK_a of 6.3 is a reasonable value for the proton dissociation from the internal aldimine. While subsequent addition of ligands to the internal aldimine (as in the case of succinate addition) will increase the ionic strength of the media slightly, the expected increase in pK_a over this range of ionic strength would, however, be predicted to be less than 0.2 units (Bergami et al., 1968).

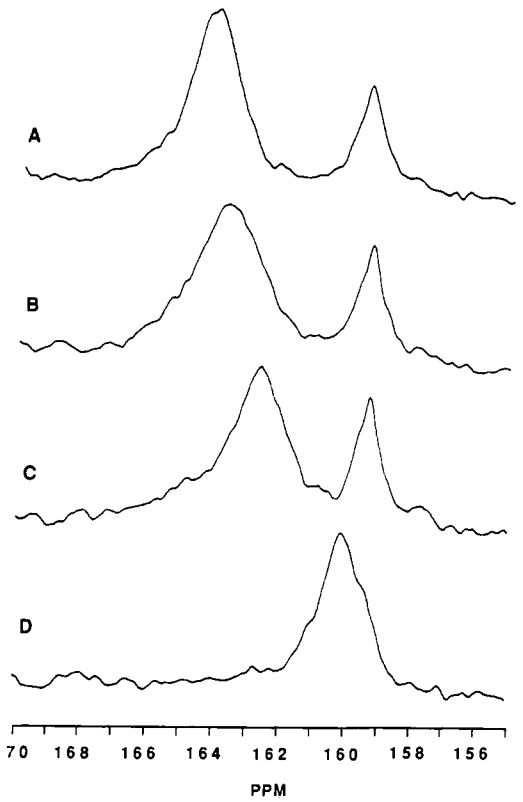


FIGURE 3: Selected region of 75.46-MHz ^{13}C NMR spectra showing the effect of pH and 2-methyl-DL-aspartate concentration on the chemical shift and line width of C4′ of the pyridoxal form of reconstituted AspAT. Protein concentration is approximately 1 mM in all samples under the following conditions of pH, MeAsp concentration, and number of scans: (A) 5.55, 0.0 mM, 65000; (B) 5.37, 10 mM, 70000; (C) 5.39, 50 mM, 75000; (D) 7.73, 0.0 mM, 290000.

of the imino group (Morino et al., 1986). The line width of C4′ during the course of the titration remained relatively unchanged, randomly varying from 115 to 140 Hz.

Effect of Succinate. The competitive inhibitor succinate has been shown to bind to the substrate binding site of the enzyme, thereby inducing a conformational change akin to substrate binding, i.e., from the open to closed conformation (Arnone et al., 1985). Thus it was of interest to test a possible effect of succinate on the ^{13}C NMR spectrum of the enzyme. The resonance line for C4′ of the coenzyme at pH 7.5 was found to shift downfield by about 4 ppm upon the addition of a saturating concentration of succinate. The effect of succinate on the C4′ chemical shift was examined further at various pH values. The titration curve (○) thus obtained yielded a pK_a value of 7.8 (Figure 2). This value is close to that proposed for the ionization of the coenzyme aldimine bond on the basis of spectrophotometric titration of the enzyme in the presence of succinate (Jenkins & D'Ari, 1966b; Metzler & Metzler, 1987). The line width of C4′ in the presence of succinate ranged from 100 ± 20 Hz at lower pH to over 150 Hz at higher pH, although estimation of the line width at higher pH is somewhat complicated due to overlap with the natural abundance signals of the protein at 160 ppm.

Effect of 2-Methyl-DL-aspartate. 2-Methyl-DL-aspartate (MeAsp) reacts with the pyridoxal form of AspAT to form a stable external aldimine complex, since the substitution of the methyl group at position 2 of the amino acid precludes further reaction leading to transamination (Fasella et al., 1966). In addition, the dissociation constant of the MeAsp–AspAT complex for the L isomer has been shown to be pH dependent, increasing sharply at low pH: 2.5–3 mM (pH 8.0–7.0), 6 mM (pH 6.0), 10 mM (pH 5.5), and 50 mM (pH

5.0) (Fasella et al., 1966). In view of the high stereospecificity of this enzyme for the L isomer of amino acid substrates, it is reasonable to assume that the enzyme preferentialy binds only the L isomer of the DL mixture employed in this study. In fact, this is fully supported by the X-ray crystallographic structure of the MeAsp–AspAT complex determined by Arnone et al. (1985b).

Figure 3 summarizes the effect of MeAsp concentration and pH on the chemical shift of the internal aldimine C4′ of AspAT. The addition of 10 mM MeAsp to the internal aldimine at pH 5.5 perturbs the C4′ resonance of the bound coenzyme from 164.5 ppm (Figure 3A) to 163.7 ppm (Figure 3B). On the basis of the above dissociation constant of 10 mM at pH 5.5, the enzyme active site should only be 33% saturated with the 5 mM L isomer of MeAsp added up to this point. Further addition of MeAsp to a final concentration (DL) of 50 mM continues to shift the C4′ signal upfield to 162.71 ppm (Figure 3C). The observed upfield shift of the C4′ signal with increasing MeAsp concentration can therefore be attributed to complex formation and not to ionic strength effects,[2] since any increase in aldimine pK_a due to ionic strength would result in a *downfield* shift of the C4′ signal at a given pH.

The observed line widths for both the internal aldimine (Figure 3A) and the external aldimine (50 mM MeAsp, Figure 3C) are comparable at pH 5.5 (130 Hz). However, the line width of the C4′ signal at 163.7 ppm in the presence of 10 mM MeAsp is observed to be 175 Hz. This increased line width present at 10 mM MeAsp can be attributed to the chemical exchange of three species: internal aldimine, Michaelis complex, and external aldimine, present at subsaturating MeAsp levels. Upon further addition of MeAsp, reduction of the line width (Figure 3C) is evident, indicating that the enzyme is more nearly saturated.

The effect of pH on the external aldimine formed in the presence of 50 mM MeAsp was next determined. As described earlier, the internal aldimine of AspAT is sensitive to pH, the C4′ resonance titrating to higher field with increasing pH (●, Figure 2). At high pH (pH 7.7, Figure 3D), the C4′ resonance is shifted 5 ppm upfield to a value of 160.2 ppm. In comparison, the AspAT–MeAsp complex at 50 mM MeAsp did not display any pH-dependent chemical shift effects, the chemical shift of C4′ remaining relatively constant at 162.7 ppm in the pH range 5–9 (□, Figure 2), in agreement with the spectrophotometric titration (Fasella et al., 1966). A slight broadening of the C4′ resonance to 175 ± 20 Hz (data not shown), however, is noted upon going to higher pH. This broadening becomes apparent at pH 6 and is not affected further on going to higher pH. No additional change in the line width or chemical shift was noted upon further addition of MeAsp to 100 mM (data not shown), indicating that the broadening seen at pH 6 and above may indicate tighter binding and further restriction in cofactor mobility at high pH. A similar effect has been noted in phosphate mobility as observed by ^{31}P NMR (Mattingly et al., 1982).

Effect of Cysteinesulfinate. Cysteinesulfinate is an amino acid substrate for AspAT. Upon transamination, it is converted to sulfinylpyruvate, which undergoes a spontaneous and rapid decomposition to release pyruvate. Since pyruvate is a poor substrate for this enzyme, the reaction is considered to be virtually irreversible and thus completely converts the pyridoxal form of AspAT to the pyridoxamine form. Addition of excess cysteinesulfinate to [4′-^{13}C]pyridoxal-P reconstituted AspAT at pH 5.15 results in rapid loss of the visible yellow color characteristic of the aldimine chromophore. The ^{13}C NMR spectra of the labeled enzyme in the presence of cysteinesulfinate indicated complete disappearance of the aldimine signal at 165 ppm (Figure 4A) with the concomitant appearance of a new signal at 39.6 ppm as revealed in the difference spectrum (Figure 4C). Under these conditions the line width of the new resonance was found to be 75 ± 10 Hz.

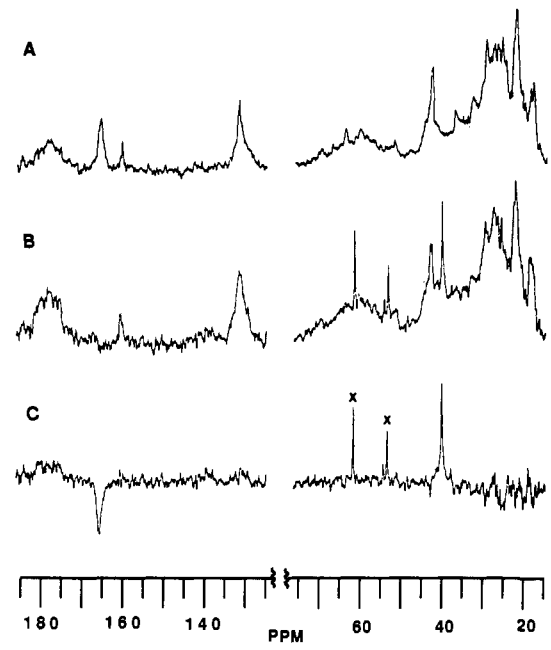


FIGURE 4: Effect of cysteine sulfinate on the pyridoxal form of reconstituted AspAT. ^{13}C NMR spectra, 125.76 MHz, of (A) 0.9 mM AspAT and 20 mM KPB, pH 5.15 (70 000 scans), and (B) after the addition of 3.3 mM cysteine sulfinate (75 640 scans); (C) represents the difference spectrum (B − A). Signals attributed to excess cysteine sulfinate are denoted by an X.

Effect of Sodium Borohydride on the Internal Aldimine of AspAT. Reducing agents such as sodium borohydride have been shown to reduce imine bonds. The [4′-^{13}C]pyridoxal-P should be especially sensitive in following the reduction of the internal aldimine of AspAT as the labeled carbon is converted from an sp^2 to an sp^3 center. Treatment of [4′-^{13}C]pyridoxal-P reconstituted AspAT at pH 6.2 with sodium borohydride resulted in a complete disappearance of the resonance at 162.65 ppm previously attributed to the internal aldimine (Figure 5A). The difference spectrum (Figure 5C) obtained between the sample before and after the addition of sodium borohydride revealed the appearance of a new signal at 47.4 ppm. The line width of the C4′ resonance upon reduction is estimated to be 250 ± 25 Hz, double that of the internal aldimine prior to reduction.

Discussion

Pyridoxal-P readily undergoes Schiff base formation with suitable aliphatic amines and amino acids. In addition, pyridoxal-P-requiring enzymes such as aminotransferases and D-serine dehydratase have been shown to form stable imines between pyridoxal-P and the ε-amino group of an active-site lysine. In this paper we have described the preparation of cytosolic AspAT reconstituted with [4′-^{13}C]pyridoxal-P and have examined the ^{13}C NMR behavior of the coenzyme C4′ under a variety of ligand conditions in order to obtain a more dynamic aspect of the interaction between the coenzyme and its surrounding environment.

^{13}C Chemical Shifts of Bound Pyridoxal-P. In aqueous solution, two chemical shifts are reported for C4′ of free pyridoxal-P (Table I) and are attributed to the aldehyde (196.8 ppm) and its hydrate (88.4 ppm). Upon reconstitution of the apoenzyme of AspAT with [4′-^{13}C]pyridoxal-P we observe at

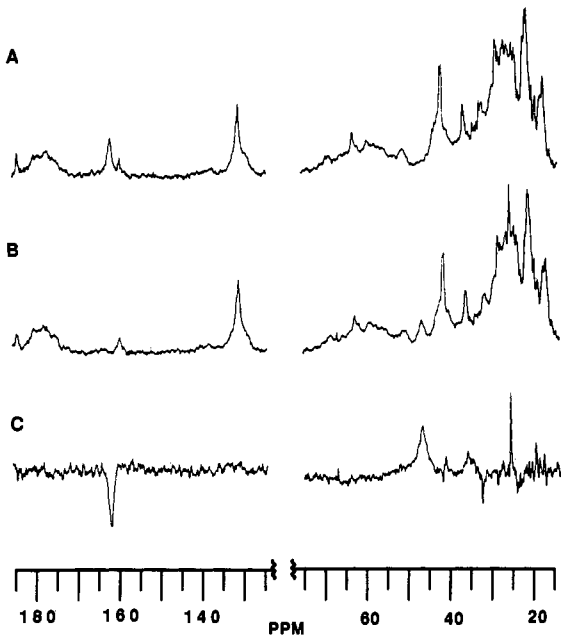


FIGURE 5: Sodium borohydride reduction of the pyridoxal form of reconstituted AspAT. ^{13}C NMR spectra, 125.76 MHz, of (A) 1.1 mM AspAT and 20 mM KPB, pH 5.8 (85 000 scans), and (B) after the addition of excess sodium borohydride (70 000 scans); (C) represents the difference spectrum (B − A).

Table I: Comparison of Chemical Shifts for C4′ of Various Derivatives of Pyridoxal-P in Enzyme-Bound and Free Form

compound	δ (ppm)	pH
pyridoxal-5′-P[a]		
aldehyde	196.8	8.4
hydrated aldehyde	88.4	8.4
aldimine derivatives		
pyridoxal-P + *n*-butylamine[a]	165.8	7.4
pyridoxal-P + valine[b]	165.3	7.6
pyridoxamine-5′-P[c]	37.6	7.0
	37.6	12.5
N-*n*-butylpyridoxamine-5′-P[a]	45.5	7.8
enzyme-bound pyridoxal derivatives		
D-serine dehydratase[a]	167.7	7.8
	168.0	9.4
reduced with $NaBH_4$	41.7	8.3
cytosolic AspAT[d]		
pyridoxal form	165.3	5.1
	160.2	9.4
complex with succinate	165.9	5.5
	164.0	7.5
complex with 2-methyl-DL-aspartate	162.7	7.5
reaction with cysteinesulfinate (pyridoxamine form)	39.6	5.1
reduced with $NaBH_4$	47.2	5.8

[a] O'Leary and Payne (1976); Jaworski and O'Leary (1979). [b] Dalling et al. (1976). [c] Mantsch and Smith (1979). [d] This study.

pH 5 only one resonance for C4′ at 165.3 ppm (Figure 1C), indicative of formation of an imine between the aldehyde of pyridoxal-P and the ε-amino group of the active-site lysine 258. The chemical shift observed at pH 5 is in good agreement with the value reported for D-serine dehydratase reconstituted with [4′,5′-$^{13}C_2$]-pyridoxal-P and is consistent with the chemical shift of a protonated imine based on model imines formed between free pyridoxal-P and various amines and amino acids (Table I). While the pK_a's of the imine nitrogen for a large group of pyridoxal-P Schiff bases are generally observed to be in excess of pH 11 (Kallen et al., 1985), the imine nitrogen of the internal aldimine of AspAT experiences deprotonation at a much lower pH (pK_a = 6.3), the C4′ resonance shifting approximately 5 ppm upfield by pH 9. The addition of succinate partially reverses this effect, increasing the pK_a of the internal aldimine to 7.8.

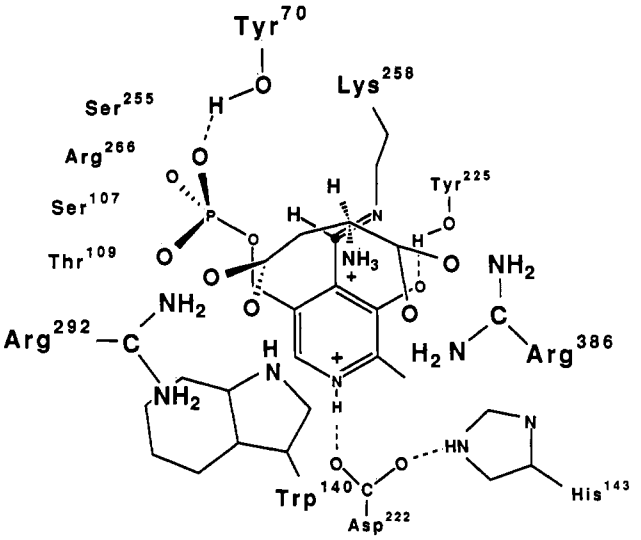


FIGURE 6: Schematic representation of the Michaelis complex of AspAT with aspartic acid highlighting the critical interactions between active-site residues with substrate and cofactor. [Estimation based on the structures of the crystalline enzyme (Arnone et al., 1985).]

The basis of the abnormally low pK_a of the internal aldimine of AspAT relative to the free coenzyme can be explained by considering the influence of the active-site environment upon the electronic structure of the coenzyme. As deduced by X-ray crystallography, the pyridoxal coenzyme is tethered to lysine 258 via the aldimine linkage with the ε-amino group (Figure 6). Key hydrogen bonds are observed between the phenolic OH of Tyr 225 with the coenzyme 3′-oxygen and between the pyridine nitrogen to the β-carboxylate of Asp 222. The latter interaction is thought to be important in stabilizing the positive charge on the pyridine ring, which has been demonstrated to lower the pK_a of the imine nitrogen. For instance, the pK_a of the Schiff base of valine and pyridoxal-P has been shown to decrease from 12.2 to 9.6 upon methylation of the ring nitrogen (Arnone et al., 1985a). Additional positive charge in the vicinity of the coenzyme is supplied by the guanidinium groups of arginines 386 and 292, which bind respectively the proximal and distal carboxylates of the substrate. The abnormally low pK of the internal aldimine in the absence of ligands may therefore reflect the propinquity of an additional positive charge in the vicinity of the coenzyme. On binding succinate, however, the carboxylic acids of the substrate effectively neutralize the positively charged guanidinium groups, shifting the pK_a of the imine to a higher value of 7.8.

The conversion of the internal aldimine of AspAT to an sp^3 center can be readily accomplished either by reduction with sodium borohydride or by conversion to the pyridoxamine form with cysteine sulfinate. Accordingly, addition of a slight excess of cysteine sulfinate to the internal aldimine of AspAT results in the complete disappearance of the ^{13}C resonance assigned to C4′ of the imine with the concomitant appearance of a new signal at 39.6 ppm, in good agreement with the value of 37.6 ppm reported to C4′ of free pyridoxamine 5-phosphate in Table I. Likewise the reduction of the internal aldimine with sodium borohydride produces a change in chemical shift comparable to the formation of pyridoxamine-P by cysteine sulfinate. Upon reduction with borohydride, C4′ of the imine is converted essentially to an N-substituted pyridoxamine, several examples of which can be found in Table I. A value of 41.7 ppm has been reported for C4′ of the coenzyme for the reduction of the Schiff base formed between [4′,5′-$^{13}C_2$]pyridoxal-P and

Table II: Effect of Ligands upon Line Width of Pyridoxal C4′ Bound to AspAT

ligand	$\omega_{1/2}$ (Hz)[a]	ligand	$\omega_{1/2}$ (Hz)[a]
none	125 ± 10	cysteinesulfinate	75 ± 10
succinate	100–150[b] ± 20	$NaBH_4$	250 ± 25
MeAsp	130–175[b] ± 20		

[a] Line width measured at half-height. [b] Variable with pH.

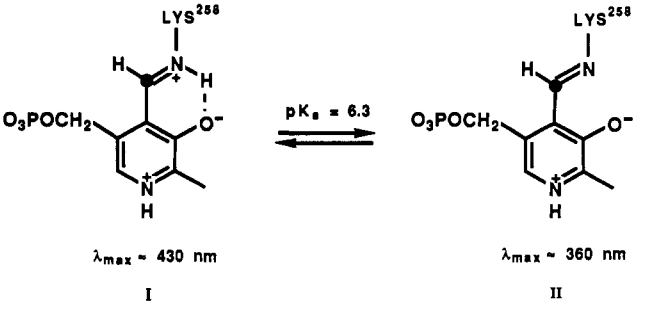
D-serine dehydratase, while in the *N*-butylpyridoxamine 5-phosphate derivative, a structural mimic of the lysine side chain, the corresponding value is 45.5 ppm. Hence the value of 47.4 ppm observed for C4′ in the reduced internal aldimine of AspAT found in this study, although perturbed downfield, falls within the expected range of chemical shift values.

Line Width of Bound Pyridoxal-P C4′. In addition to the obvious chemical shift data obtained in a ^{13}C NMR measurement, the line width of the individual resonances can convey important information regarding the molecular dynamics of the protein [for a detailed description of ^{13}C NMR of proteins see reviews of Malthouse (1986) and Allerhand (1979)]. The observed line width of a ^{13}C nucleus in a macromolecule is governed primarily by ^{13}C–^{1}H dipolar relaxation and the molecule's rotational correlation time (τ_r), a measure of the molecule's rate of tumbling. However, the internal rotations of isolated nuclei can be faster than the overall tumbling rate of the enzyme ($\tau_{eff} > \tau_r$) and in this situation the observed line width will be decreased substantially. Accordingly, line-width analyses can be important in determining the degree of binding or motional freedom of individual carbons of interest.

Based on the estimated rotational correlation time of 130 ns for AspAT (Churchich, 1967) a rigidly bound carbon containing one attached proton can be estimated to have a maximum line width of approximately 175 Hz. The observed line widths for C4′ of the coenzyme of AspAT under a variety of conditions are summarized in Table II. In the pyridoxal form of the coenzyme, bound as the internal aldimine with lysine 258, the line width for C4′ was observed to be 125 Hz, suggesting the coenzyme to be tightly bound with only a small degree, if any, of rotational freedom above the overall tumbling rate of the protein. The addition of the ligand succinate at alkaline pH elicits a slight increase in the line width of C4′, which may be indicative of the conformational closing of the active site induced by the presence of succinate. In a similar fashion the binding of 2-methylaspartate at pH 6 and above increased the line width of C4′, despite the loss of the coenzyme's covalent tether to lysine 258. This result is consistent with the strong influence of the ionic interactions between the carboxylates of the substrate and the guanidinium groups of arginines 292 and 386 as well as the active site closing around the substrate as deduced by X-ray crystallography. An alternative explanation for the increased line width observed may be the preponderance at higher pH of several distinct MeAsp–AspAT complexes (discussed further below) occurring near an intermediate rate of exchange.

The conversion of the aldimine forms of AspAT to the pyridoxamine form either by reaction with cysteine sulfinate or by reduction with sodium borohydride changes C4′ of the coenzyme from an sp^2 to an sp^3 center. As mentioned above, ^{13}C–^{1}H dipolar relaxation is the other major factor influencing ^{13}C line widths in macromolecules, and as a result ^{13}C line widths will depend on the number of protons directly attached to the nucleus being observed. Therefore, the addition of a second proton can be expected to double the maximum line width of C4′ to a value of 350 Hz. The line width of C4′ in the internal aldimine upon reduction with borohydride (Table II) reflects the predicted increase in line width, doubling to a value of 250 Hz. This values suggest that overall no additional freedom of motion is gained on reduction. In contrast, the observed line width of 75 Hz for C4′ in the pyridoxamine form (via cysteine sulfinate treatment) reflects a *decrease* in line width, indicating that the C4′ of the coenzyme has gained additional rotational freedom upon conversion to the pyridoxamine form. While pyridoxamine-P is bound somewhat less tighly than pyridoxal-P, its binding constant is still in the micromolar range, suggesting that the decrease in line width of C4′ in the pyridoxamine form is reflective of *localized* increased motion of the aminomethyl side chain, not of the coenzyme on the whole.

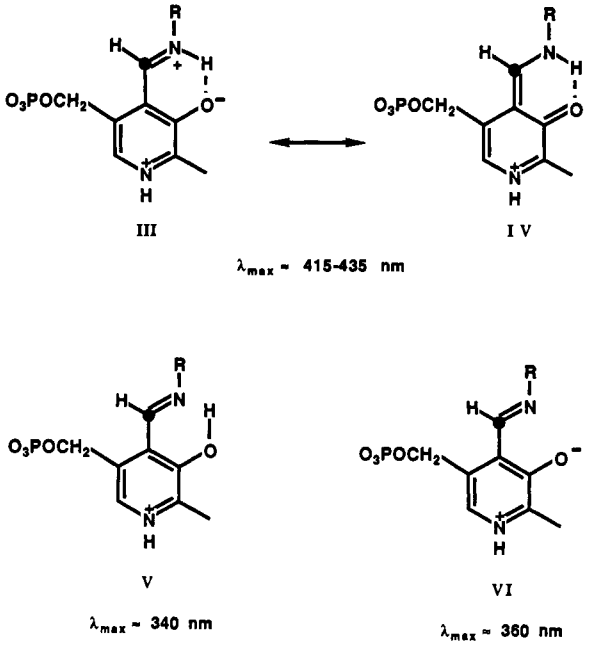
The Nature of the MeAsp Complex. The interaction of 2-methylaspartate with the internal aldimine of AspAT goes one step beyond succinate binding as the ϵ-amino group of lysine 258 is replaced by the amino group of MeAsp in a reversible "transaldimation" reaction. Spectrophotometric observations of the conversion of the internal to the external aldimine show the production of two absorption bands at 430 and at 360–370 nm of roughly equal intensity. This is similar in appearance to the components of the internal aldimine absorption spectra, which display a low-pH maxima at 430 nm and a high-pH maxima at 360 nm attributed respectively to the protonated (I) and unprotonated (II) forms of the aldimine. Unlike that of the internal aldimine, however, the



I II

absorption spectra of the external aldimine is shown to be insensitive to pH over the range pH 5–9 (Fasella et al., 1966), a result that has now been confirmed by ^{13}C NMR spectroscopy. At saturating concentrations of DL-MeAsp (>50 mM), the chemical shift of C4′ of the complex formed was found to remain constant at 162.7 ppm over the pH range 5–9.

The insensitivity of the external aldimine chromophore to pH, despite its similarity in appearance to the internal aldimine, is not well understood. Detailed spectrophotometric studies indicate that Schiff bases of free pyridoxal-P at low and neutral pH can exist in several different tautomeric forms. The ketoenamine form, which is composed of the resonance structures III and IV, absorbs maximally between 415 and 435 nm and predominates in aqueous solution of high polarity, while the enolimine structure (V) absorbs maximally at 340 nm and is favored in solvents of low polarity (Kallen et al., 1985). Although ketoenamine structures III and IV are functionally different about C4′, they are shown to display similar downfield ^{13}C chemical shifts for C4′ of 165 ppm and above (O'Leary & Payne, 1976). Likewise the chemical shift of C4′ in the enolimine form (V) would be predicted to be quite similar to the upfield shift of 160 ppm for the unprotonated dipolar ion observed in this study. The present finding that the chemical shift of C4′ in the presence of MeAsp is located at a position intermediate between these two extremes supports the existence of two (or more) rapidly equilibrating species, one absorbing spectrally at 430 nm and the other at 360–370 nm. In addition, the minor increase in line width observed



upon formation of the external aldimine complex with MeAsp lends additional support for the existence of an equilibrium process.

Scott et al. (1985) reported a similar observation in examining the ^{19}F NMR of porcine cAspAT reconstituted with 6-fluoropyridoxal phosphate (FPLP). In this study, substitution with the electronegative fluorine was noted to invert the tautomeric preference of the internal aldimine at pH 5.4 from predominantly the ketoenamine (430 nm) form observed in the native enzyme to mainly the enolimine (340 nm) form. Addition of MeAsp led to a reversal of this tautomeric preference, with the ketoenamine form now predominating over the enolimine by roughly 2 to 1. More importantly, the ^{19}F chemical shift observed in the presence of MeAsp was found to be intermediate between the ^{19}F chemical shifts predicted for the enolimine and ketoenamine tautomers, although closer to the chemical shift of the ketoenamine, reflecting the higher percentage of ketoenamine component seen in the absorption spectra. It was concluded that the position of the ^{19}F chemical shift of FPLP in the presence of MeAsp reflected a rapid equilibrium between the ketoenamine and enolimine tautomers. In contrast, the enolimine tautomer makes up only a minor component (less than 20%) of either the internal or external aldimines of native cAspAT at low pH. The spectral studies and our ^{13}C chemical shift data of the external aldimine complex with MeAsp suggest a equilibrium between roughly equal populations of ketoenamine and dipolar ion species.[3]

The origin of the ketoenamine component can be assigned with reasonable certainty to the MeAsp external aldimine complex. However, two possibilities exist for the origin of the dipolar ion species. First, the 360–370-nm absorption band may represent the unprotonated dipolar ion of the external aldimine VI analogous to that of the internal aldimine II. In this case however, it would be difficult to explain how the requisite interconversion between the ketoenamine and dipolar forms of the external aldimine could remain independent of pH. A second and perhaps more likely explanation is that the dipolar ion observed is the Michaelis complex between MeAsp and the internal aldimine. In addition to contributing the proper absorption spectra and ^{13}C chemical shift components to the equilibrium mixture observed, the Michaelis complex might also be expected to display the pH sensitivity observed of dissociation constant of the complex. Indeed the rapid increase in the dissociation constant of the MeAsp–AspAT complex (Fasella et al., 1966) roughly parallels the pK_a of the internal aldimine, suggesting that protonation of the imine produces unfavorable charge repulsion, hence dissociation, between the aldimine and amine of MeAsp. It is of interest to note that in the case of FPLP AspAT, which at low pH exists predominantly as the enolimine tautomer and not the ketoenamine, appears to display a much lower dissociation constant for the MeAsp complex (Scott et al., 1985).

While it is not possible with the present results to determine unequivocally the precise nature of the dipolar ion species observed for the MeAsp complex, it is interesting to speculate on the nature of the equilibrium mixture observed; i.e., does the mixture represent equivalent subunits, each in a state of equilibrium between the ketoenamine and dipolar ion species, or does the complex exist simultaneously as the protonated external aldimine in the active site of one subunit and *either* the unprotonated external aldimine complex *or* the Michaelis complex of the unprotonated internal aldimine in the adjacent subunit? Although such functional nonequivalence between the two subunits it not observed in the experiments using natural substrates in solution (Boettcher & Martinez-Carrion, 1976), nonequivalence is observed in the microcrystalline enzyme with both natural substrates and inhibitors (Christen & Kirsten, 1985) as well as in the X-ray structure of the MeAsp complex (Arnone et al., 1985b). The tertiary structure as defined by the crystallographic data exhibits only one subunit bound with MeAsp in the closed conformation, while the other subunit remains unoccupied in the open conformation. In addition, in the unliganded native enzyme there is a water molecule in position to interact with the internal aldimine bond, while in the MeAsp complex, upon closure of the active site, there is no evidence for a water molecule near the aldimine bond (Arnone, personal communication). This fact supports the contention that the external aldimine bond in the complex may be insulated from the solvent and thus its ionization state is rendered insensitive to the change in solvent pH.

To conclude, in this paper we have described the first application of ^{13}C NMR spectroscopy to the study of the pyridoxal-P-requiring aminotransferases. The results clearly demonstrate the feasibility of utilizing selective ^{13}C enrichment to probe the mechanism and local environment of macromolecular enzymes approaching 100000 MW. In this case, pyridoxal coenzyme labeled at C4′ was incorporated into the active site of AspAT and shown to be sensitive to the mechanistic and structural changes associated with ligand binding. Additional experiments can now be devised involving ^{13}C-enriched substrates or inhibitors as well as using site-specific mutagenesis to further probe the mechanism of AspAT via ^{13}C NMR spectroscopy.

Registry No. AspAT, 9000-97-9; MeAsp, 866-73-9; pyridoxal-P,

[3] It has been pointed out that if the MeAsp–AspAT complex represents an equilibrium mixture of two species undergoing exchange, the chemical shift and line width of the C4′ resonance may exhibit temperature-dependent changes. If one assumes the chemical shifts of the exchanging species as 166 and 160 ppm (i.e., from the ketoenamine and dipolar ion species, respectively), then at 75.46 MHz $\Delta\nu$ = 450 Hz and, from $k = \pi\Delta\nu/2^{1/2}$, the rate of exchange (k) near coalescence would be approximately 1000 s^{-1}. If the observed increase in line width in the presence of MeAsp is due solely to exchange broadening, the rate of exchange above coalescence can be estimated from $k = \pi\Delta\nu^2/2\omega$, where ω is the increase in line width (25–50Hz) due solely to the exchange broadening, and then the observed rate at 20 °C would be in the range 5000–10000 s^{-1}. Based upon these estimates it may indeed be possible to induce substantial changes of C4′ line width with varying temperatures. The feasibility of this experiment is currently under investigation.

54-47-7; L-lysine, 56-87-1; succinate, 110-15-6; L-cysteinesulfinate, 1115-65-7.

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