31P Nuclear Magnetic Resonance of Mitochondrial Aspartate Aminotransferase

THE EFFECTS OF SOLUTION pH AND LIGAND BINDING*

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Mary E. Mattingly, Joseph R. Mattingly, Jr., and Marino Martinez-Carrion
From the Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

1-Aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1) of higher organisms exists as cytoplasmic and mitochondrial isozymes with a high degree of sequence homology. This report describes an NMR study of the environment of the phosphate of the pyridoxal 5'-phosphate cofactor in the mitochondrial isozyme as revealed by the effect of pH on its 31P NMR chemical shift. Our previous study of the cytoplasmic isozyme revealed a phosphate resonance which was unaffected by pH (Martinez-Carrion, M. (1975) Eur. J. Biochem. 54, 39-49). In contrast to this apparently static environment, the cofactor phosphate resonance of the mitochondrial isozyme is affected by pH, and this effect is different in the various enzyme forms previously identified as intermediates in the catalytic sequence of transamination. The effect of pH is described by a pK of 5.5 and by limiting chemical shifts of -2.4 and -3.5 ppm for the cofactor phosphate in the deionized enzyme. Chloride binds to the deionized enzyme, increasing the pK of the cofactor phosphate to 6.3. The substrate analogue a-methyl-L-aspartate broadens the resonance of the cofactor phosphate and results in a chemical shift constant at -3.8 ppm from pH 8.5 to about pH 6, decreasing slightly at lower pH. The substrate analogue erythro-beta-hydroxy-L-aspartate produces two overlapping 31P resonances. By contrast to all other forms of this isozyme, the phosphopyridoxamine form of the enzyme has a chemical shift which is unaffected by pH, remaining at -2.3 ppm. Succinate, a possible regulatory ligand which inhibits the enzyme, causes an increase in the cofactor phosphate pK to 7.8 and in the limiting chemical shifts to -3.1 and -3.9 ppm. The apotransaminase tightly binds inorganic phosphate, but it can be displaced by arsenate. The bound phosphate shows a chemical shift behavior somewhat similar to that of the phosphate ester of the bound cofactor. These observations are discussed in terms of the diversity between the isozymes of aspartate aminotransferase and the possible roles of the cofactor phosphate and its protein environment.

Pyridoxal 5'-phosphate participates in many enzymic reactions which can be categorized according to the following schemes in which the cofactor functions. Glycogen phosphorylases are enzymes in which pyridoxal-P* was first thought to fulfill a structural role but is now thought to participate in catalysis through its phosphate moiety (1, 2). However, in the majority of pyridoxal-P-dependent enzymes, the cofactor forms covalent aldimine or ketimine adducts with substrates, activating the substrate for subsequent reactions. In all cases, the phosphorylated cofactor is necessary for efficient catalytic activity, but the precise functions of the phosphate group are unknown.

31P NMR is a well suited probe for the environment of phosphate since the factors which effect 31P chemical shifts are relatively well characterized (3). The 31P magnetic resonance frequencies of pyridoxal-P and its derivatives vary with pH; this observation has been used to assess the ionization state of the phosphate when the cofactor is bound to its functional site (4, 5).

The enzymes utilizing pyridoxal-P in covalent catalysis yield a variety of reactions, and the 31P NMR results observed in the systems examined to date have also proved diverse. The cytoplasmic isozyme of aspartate aminotransferase (EC 2.6.1.1) was first examined (4). The 31P resonance of the cofactor, whether covalently bound to the protein as the internal aldimine or as pyridoxamine-P, appeared to be unchanged by pH or by the presence of ligands. The chemical shift was consistent with a dianionic form of the phosphate group. On the other hand, the phosphate of the cofactor of p-serine dehydratase was found to show a chemical shift dependence on pH (5). Furthermore, in the binary complex with the competitive inhibitor isoserine, the 31P chemical shift was unaffected by pH; the invariant chemical shift was also indicative of a dianionic phosphate ester. Tryptophanase was recently reported to behave similarly to cytoplasmic aspartate transaminase; the cofactor remained in the dianionic form over the accessible pH range, and the resonance was unaltered by the presence of substrate. On the other hand, in serine hydroxymethyltransferase, the 31P resonance of the cofactor phosphate is affected by pH, but this behavior is unaltered by ligands.5 This report describes a 31P NMR study of the mitochondrial isozyme of aspartate aminotransferase. This enzyme has a 48% amino acid sequence homology to the cytoplasmic isozyme (6), and crystallographic studies have implied a degree of structural similarity in low resolution and unrefined electron density maps of the two isozymes, including the regions accommodating the cofactor phosphate ester (7). This study, however, reveals pronounced differences in the protein envi-

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1 The abbreviations used are pyridoxal-P, pyridoxal 5'-phosphate; pyridoxamine-P, pyridoxamine 5'-phosphate.

2 K. D. Schnackerz, results presented at the Pyridoxal Phosphate Associated Enzymes Symposium, June 1981, Knoxville, TN.

3 Quashnock, L. Schirch, and J. Chlebowski, results presented at the Pyridoxal Phosphate Associated Enzymes Symposium, June 1981, Knoxville, TN.
The mitochondrial isozyme was similarly purified from porcine heart aspreviously described (8, 9). The mitochondrial isozyme was similarly purified from porcine heart by a procedure slightly modified from that previously published (10). The latter preparations had specific activities of approximately 200 units/mg, using a coupled assay (11). The protein concentration was determined spectrophotometrically using ε = 130,000 cm⁻¹ M⁻¹ for a dimer of subunit molecular weight of 44,647 (12). The pyridoxal-P content of the enzyme was measured spectrophotometrically after the addition of NaOH to 0.1 N using ε = 6500 cm⁻¹ M⁻¹ at 388 nm (13); the ratio of pyridoxal-P/subunit was 1.00 ± 0.05. The pyridoxamine-P form of mitochondrial aspartate transaminase was prepared by reaction with cysteine sulfinate (14). The apoenzyme was prepared as previously described (15). Before use in the NMR measurements, the enzyme was extensively dialyzed against 0.1 M KCl containing 1 mM EDTA, pH 7, with NaOH and 0.02% sodium azide. In studies on the effects of chloride and succinate binding, the KCl was omitted. The enzyme was concentrated by ultrafiltration.

The a-subform of the cytoplasmic isozyme of aspartate aminotransferase was purified from porcine heart as previously described (8, 9). It is well known that in aspartate aminotransferase a variety of enzyme-substrate and enzyme-inhibitor complexes can be detected through the absorption spectral properties of the active site chromophore, pyridoxal-P. The complexes in Scheme 1 are indicative of the nature and properties of these intermediates in the transamination sequence.

**MATERIALS AND METHODS**

**Pyridoxal-P Form of the Aminotransferase in the Absence of Ligands**—The mitochondrial isozyme of aspartate aminotransferase shows a single 31P resonance, the chemical shift of which varies with pH (Fig. 1). The data described by the curve •—• were obtained using an enzyme that was extensively dialyzed against dilute Tris-cacodylate buffer. The range of pH values accessible for examination is limited by the stability of the enzyme. No enzymic activity is lost in the course of data acquisition between pH 5 and 9; the titration is also completely reversible between these extremes. The pH dependence over this range can be described as the ionization of a simple monoprotic acid. Curve •—• in Fig. 1 is a theoretical fit for a group with a pK of 5.5 ± 0.1 and chemical shifts of -3.5 ± 0.1 ppm for the species present at high pH and -2.4 ± 0.1 ppm for the species at low pH. The phosphate pK of the model Schiff's base of pyridoxal-P and e-aminoacetate is 6.3 (5) and that of free pyridoxal-P is 6.2 (4). The chemical shift of the model Schiff's base is about -0.6 ppm at low pH and about -3.8 ppm at high pH. Free pyridoxal-P has phosphate chemical shifts of about 0 ppm and -3.8 ppm at low and high pH, respectively. The line width of the bound cofactor is about 14 ± 1 Hz in the presence of broad band proton decoupling; it does not change significantly with pH.

![Scheme 1](image-url)
The Effect of pH on the 31P NMR chemical shift of mitochondrial aspartate aminotransferase. The symbols used are: •, the aldimic form of the enzyme alone; ○, enzyme in 0.1 M KCl; ▲, enzyme in 95 mM succinate; ▣, enzyme in 95 mM dl-a-methylaspartate; ■, enzyme in which the cofactor was converted to the pyridoxamine-5'-phosphate form. The datum indicated by the arrow results from the addition of 5 mM a-ketoglutarate to the pyridoxamine form. The enzyme concentration was approximately 2 mM in all these experiments.

This line width is not detectably altered in the absence of proton decoupling.

**The Effect of Chloride**—A variety of ligands interact with the aminotransferase. The addition of 0.1 M chloride alters the effect of pH on the chemical shift of the phosphate of the aldimic cofactor, as also shown in Fig. 1. The pK is increased, and a more complete titration curve is obtained. Curve ○—○ in Fig. 1 is drawn for a single ionization with a pK of 6.3 ± 0.1 and limiting chemical shifts of −3.6 ± 0.1 ppm at high pH and of −2.3 ± 0.1 ppm at low pH. The presence of chloride does not alter the line width of the resonance, which remains at about 14 ± 1 Hz. No further effect is seen at a 5-fold higher concentration of chloride. These concentrations of chloride do not alter the titration curve of the free cofactor.

**The Effect of Succinate**—Certain dicarboxylic acids bind to the aldiminic enzyme to form abortive complexes. Because of the high affinity and the chemical stability of succinate, it was examined for its effect on the pH dependence of the phosphate of the aldimic cofactor, as also shown in Fig. 1. Above pH 6, the chemical shift is constant at about −3.8 ppm, similar to that observed at high pH in the presence of succinate. Only at values lower than pH 6 does the chemical shift decrease. The line width is increased to approximately 27 ± 3 Hz by a-methyl-L-aspartate. At 12 mM a-methyl-L-aspartate, the line widths and the end points of the calculated titration curve are similar to those of the enzyme in the absence of the ligand; however, the pK is about 0.5 lower than the expected value.

The Effect of erythro-β-Hydroxy-L-aspartate—erythro-β-Hydroxy-L-aspartate induces an almost complete transamination half-reaction. The addition of a 5 mM concentration is accompanied by the rapid appearance of an intense absorbance of the enzyme at 498 nm and less intense bands at 392 and 330 nm. This spectrum undergoes a very slow decrease in absorbance at 392 and 392 nm to produce an increase in absorbance at 330 nm; however, the absorbances at 498 and 392 nm decrease only 6% of their initial value over the 18 h necessary to collect the NMR data. The addition of sodium hydroxide to the enzyme-erythro-β-hydroxyaspartate complex present at the termination of data collection produced the absorption peak at 388 nm characteristic of the free pyridoxal-P cofactor. The intensity of this band indicates that little of the aldimic cofactor was converted to pyridoxamine-P.

As indicated in Fig. 2, NMR spectra were measured at pH 5.13, and 5.82. Both spectra showed overlapping 31P resonances of unequal intensity. The downfield peak of lesser intensity had a chemical shift of −4.2 ± 0.2 ppm at pH 5.13 and −4.3 ± 0.2 ppm at pH 5.82 and appeared somewhat broader than the upfield peak. The more intense upfield peak had a chemical shift of −2.9 ± 0.2 ppm at pH 5.13 and −2.9 ± 0.2 ppm at pH 5.82.

The Pyridoxamine-P Form of the Enzyme—In transamination, an amino acid substrate reacts with the aldimic transaminase to give a substrate-cofactor aldime. The complex with a-methylaspartate is a model for this intermediate. A prototropic rearrangement follows, proceeding through a semiquinoid intermediate to yield a ketimine. The interaction of the enzyme with erythro-β-hydroxyl-α-aspartate is a possible model for this stage of catalysis. The ketimine undergoes hydrolysis to produce the keto acid product and the aminic form of the transaminase. This complex of pyridoxamine-P with the apoprotein can be studied directly. Pyridoxamine-P is not bound as tenaciously as the aldimic cofactor, yet removal of the cofactor still requires a low pH and high concentrations of salts of divalent anions. By contrast to the aldimic cofactor, the 31P resonance of the aminic form of the bound cofactor is insensitive to changes in the solution pH (Fig. 1). The chemical shift, −2.3 ppm, is similar to that observed at low pH for the aldimic enzyme. Free pyridoxamine-P titrates normally with a pK of 5.6 and chemical shifts of −3.4 ppm for the dianionic species and 0 ppm for the monoanionic species. The width of the phosphate line of the enzyme-bound pyridoxamine cofactor is about 14 Hz. α-Ketoglutarate is known to convert the aminic enzyme to the aldimic form; and, as shown in Fig. 1, the addition of α-ketoglutarate to the pyri-
doxamine-P form of the enzyme at high pH results in an increase in the chemical shift to the value expected for the aldiminic form of the enzyme.

The Effect of Sodium Borohydride—The reaction of the aldiminic enzyme with sodium borohydride inactivates the enzyme through the reduction of the internal Schiffs base. This reduction also dramatically increases the pK value of the phosphate ester to 8.3 ± 0.1 (Fig. 3). The chemical shift of the species at low pH, 1.6 ± 0.1 ppm, is decreased with respect to the other enzyme-bound phosphopyridoxyl examples; the chemical shift of the species at high pH, however, is approximately the same (~3.6 ± 0.1 ppm) as that of the nonreduced Schiffs base. The line width remains unchanged at 14 ± 1 Hz after reduction.

Apoenzyme—Under appropriate conditions, the cofactor can be removed from the enzyme. The standard final step of apoenzyme preparation is dialysis against high concentrations of phosphate buffer at low pH, but, even after subsequent extensive dialysis against 10 mM Tris-cacodylate buffer, pH 8, containing 0.1 mM KCl and 1 mM EDTA, a 31P resonance is still observed in the enzyme sample. The pH dependence of the chemical shift of the line (Fig. 4) is dissimilar to that observed for the holoenzyme forms or for free inorganic phosphate. The addition of 0.1 mM L-aspartate alters the effect of pH on the chemical shift (Fig. 4). The line width in the presence or absence of substrate is about 14 Hz. Further dialysis of this apoenzyme against 10 mM sodium arsenate, pH 8, containing 0.1 mM KCl eliminates the 31P NMR signal from the apoenzyme. The addition of a stoichiometric amount of pyridoxal-P restores full enzymatic activity and a 31P resonance which behaves as shown in Fig. 1 for holoenzyme.

Effect of Mn2+ on 31P Resonance of the Enzyme—Mn2+ has been useful as a paramagnetic relaxation probe in NMR studies of the degree of exposure of enzyme-bound phosphoryl groups (17). The effect of pH on the chemical shift of the phosphate of enzyme-bound pyridoxal-P could imply an accessibility to solvent in at least the mitochondrial isozyme. Yet, the line widths of the 31P resonances of both isozymes are unaffected by Mn2+ concentrations up to 0.3 mM at an enzyme concentration of 3 mM. For a group easily accessible to Mn2+, line broadening should be detected at much lower concentrations of Mn2+.

**DISCUSSION**

The high degree of sequence homology reported between the isozymes of aspartate aminotransferase is thought to be elaborated in a high degree of similarity in their tertiary structures (7). Despite the similarities between isozymes, there are substantial differences which may provide insights into the fine detail of the enzyme mechanism. The environment provided for the phosphate of the cofactor appears to be among the fine distinctions between isozymes.

Only in the mitochondrial isozyme is the chemical shift of the phosphate of the aldiminic cofactor perturbed by changes in pH, while in the cytoplasmic isozyme the phosphate chemical shift is unaffected by pH (4). The pH sensitivity found in the mitochondrial enzyme could imply that the phosphate is accessible to the solvent. However, the inability to observe paramagnetic relaxation upon the addition of a high concentration of Mn2+ to either isozyme suggests that access to the phosphate is somewhat restricted in both enzymes. The crystal structure of both isozymes shows the cofactor lying within a deep cleft (18, 19). This is consistent with the inability of the large hexasaquomanganese (II) species to approach the phosphate, since the Mn−H radius is approximately 2.8 Å. In this sense, aspartate aminotransferase is similar to glucose oxidase in that a covalently bound phosphate on the exterior of the oxidase is accessible, while the pyrophosphate ester of the FMN cofactor is not accessible (17).

The variation of chemical shift between two pH extremes can imply that an unprotonated and a protonated species may exist. This is the simplest interpretation for the effect of pH on the phosphate chemical shift of the free cofactor and probably also applies to the bound cofactor. The chemical shift of the phosphate of enzyme-bound pyridoxal-P at high pH is very similar to that of the ε-aminoacproate pyridoxal-P Schiff's base and presumably reflects the presence of a bound dianionic phosphate ester. As the pH is decreased the line moves upfield for both the free and the bound cofactor, suggesting the presence of a bound monoanionic phosphate ester. However, the chemical shift of the compound increases ~4 ppm, whereas the chemical shift of the bound phosphate ester increases only ~2 ppm. This difference in chemical shift between the free and bound monoanionic form of pyridoxal-P could be the result of some steric constraint imposed by amino acid residues at the active site.

Though several theoretical explanations of 31P chemical shifts have been advanced, none are universally applicable to all phosphorus compounds (3). An empirical relation between the O−P−O bond angle and the phosphorus chemical shift has been advanced for phosphates (21). In general, distortions from tetrahedral symmetry which decrease the O−P−O bond angle result in a downfield shift of the phosphate in various esters. Conversely, molecules with distortions increasing the O−P−O bond angle have chemical shifts upfield. The changes in O−P−O bond angle observed in the crystal structures and used as the basis for this correlation are the result of either intramolecular steric constraints or the extent of phosphonate ionization. For instance, in the 5-membered cyclic phosphate diesters examined, the bond angle is reduced to ~96°8°, and the chemical shift is ~10−20 ppm downfield from phosphoric acid. In unstrained monoesters, the O−P−
O bond angles are similar for both the free acid and the monooanion (−103°−104°), and the chemical shifts are similar (−0 ppm). Ionization to the dianion decreases the bond angle to about 102° with an accompanying 3−4 ppm downfield shift.

Consequently, the effect of pH on the cofactor phosphate chemical shift could be a combination of the extent of protonation of the phosphate and distortion of the O−P−O bond angle. The cofactor phosphate is also bound to the enzyme through electrostatic interactions of the negatively charged phosphate to positively charged moieties contributed by the protein matrix. There is some evidence suggesting that the cofactor is bound more tightly to the enzyme if the phosphate is present as the dianion. For instance, the removal of the pyridoxamine-P of the cofactor utilizes a low pH, which favors a monooanionic phosphate. The relative importance of the stabilizing interaction of the cofactor phosphate and the protein matrix is probably increased since the covalent linkage between enzyme and cofactor is not present, and yet the enzyme still has a great affinity for pyridoxamine-P. These electrostatic interactions could account for the perturbations in pK observed for the phosphate of the bound cofactor as well as provide the source of steric constraints which could alter the chemical shift of the monooanionic or dianionic phosphate moiety.

The chemical shift of the phosphate of the cofactor in the cytoplasmic isozyme of the transaminase was not only unaffected by pH, it was also insensitive to the addition of enzyme ligands (24). By contrast, in the mitochondrial isozyme, the pH effect on the cofactor phosphate is altered in dramatic fashions by enzyme ligands. Increasing the concentrations of ligands beyond that known to fully occupy the enzyme-binding sites results in no further alterations in the titration behaviors. This saturation effect seems consistent with perturbations in pK values and end points by specific ligand binding to the enzyme, not simply an effect of solution ionic strength. Furthermore, the pH dependence of the phosphate chemical shift of the free cofactor is unaffected by these concentrations of ligands.

The simplest ligands, anions such as chloride, acetate, and phosphate, have limited effects on the properties of the transaminase. These ligands are competitive inhibitors of substrate binding and occupy protein-binding sites which may accommodate the carboxylate portion of the substrates or simply stand in the way of substrate and its active site pocket (22). The electronic transition spectrum of the aldiminic transaminase is sensitive to pH, exhibiting a hypochromic absorption shift which is thought to be due to deprotonation of the iminium nitrogen of the internal aldimine (23). The pK of this active site chromophore is increased in binary complexes with these anion ligands in what is likely an electrostatic effect. Yet, the gross structural features of the enzyme appear unaltered, as reflected by the negligible effect of these ligands on the enzyme’s thermal stability. It is of interest that the effect of Cl− on the pH dependence of the chemical shift of pyridoxal-P is similar to its effect on the pK of the active site chromophore. In the presence of Cl−, the pK of the phosphate is 6.3 and that of the chromophore is also about 6.3. In the absence of Cl−, the pK of the phosphate is 5.5 and that of the chromophore is 5.4. The chemical shifts at the pH extremes are the same regardless of the presence of chloride. This could imply that a similar degree of O−P−O bond angle distortion exists in the binary complex with Cl− and in the enzyme alone. It is not possible to ascertain from these experiments whether the effect of Cl− on the pK of the phosphate is direct or whether the effect is transmitted through its effect on the ionization of other enzyme residues. 4- and 5-carbon dicarboxylic acids bind to both aminotransferases, forming abortive complexes (Scheme 1) which increase the thermal stability of the enzyme. This has long been utilized in the purification of this enzyme and has been quantitatively confirmed in recent studies (24) for the cytoplasmic enzyme which suggest that the protein structure becomes more rigid in the presence of these ligands. The pK of the active site chromophore is also increased dramatically by these ligands; the chromophore pK of the binary complex with succinate is 8.3. The effects of succinate also extend to the environment of the phosphate of the cofactor. The pK appears increased to about 7.5, consistent with the large effect of succinate on the pK of the active site chromophore. The chemical shift at high pH, −3.9 ppm, is greater than that of the enzyme without succinate, −3.5 ppm, and is slightly greater than that of the dianionic phosphate of the free cofactor, perhaps suggesting a small decrease in O−P−O bond angle in the succinate complex. At low pH, the chemical shift is increased 0.7 ppm over that found for the enzyme alone. This could imply greater steric constraints upon the monooanion of the cofactor phosphate in the enzyme-succinate complex, as one might have inferred from the stabilizing effects of succinate on the overall enzyme structure. At this low pH, succinate is thought to act as an ionic cross-link between a protonated group in the protein and the aldimine nitrogen (24).

In the actual half-reaction of a-amino acid and the internal aldimine going to keto acid and the pyridoxamine-P form of aspartate aminotransferase, the reaction occurs through the initial transamination step of transaldimination to form an external aldimine intermediate. The reaction can proceed further since the inert methyl group replaces the labile proton of a competent substrate. There are ample reasons to assume that α-methylaspartate is not a good substrate analogue insofar as its effects may be atypical of those induced by normal substrates (24). At basic pH, the binding has been interpreted as the formation of two complexes, one of which has absorption spectral characteristics similar to those of the internal aldimine (25). In the presence of α-methylaspartate, the rate of the reaction of a specific cysteinyl residue (Cys-166) with various modifying reagents is considerably enhanced by this analogue (26), though cysteine-166 is well removed from the active site (7). Differential scanning calorimetry reveals an unusually large stabilization against thermal denaturation for both the cytoplasmic (24) and mitochondrial isozyme. The conformational change imputed in these observations has also been recently claimed from crystallographic studies (27, 28). However, in these crystals, α-methylaspartate is thought to bind to only one subunit.

The effects of α-methylaspartate on the pH dependence of the chemical shift of the cofactor phosphate are complex. Initial studies showed a 10-fold reduction in the apparent affinity of the cytoplasmic isozyme for α-methylaspartate at low pH (25). However, the buffers contained anions later shown to compete with substrate binding (29). In the absence of anions with a high affinity for the cytoplasmic isozyme, the true dissociation constant for α-methylaspartate is independent of pH from about 5.5 to 5.5. The effect of pH on the true dissociation constant has not been studied for the mitochondrial isozyme; however, it is probably also independent of pH. The phosphate chemical shift decreases only at the lowest pH.

A. Iriarte, J. F. Chlebowski, and M. Martinez-Carrion, manuscript in preparation.
values, at which the effect of pH on the binding of α-methylaspartate has not been studied. This decreasing chemical shift could possibly be the result of an increasing proportion of free enzyme at this low pH; more likely, though, is that the pK of the phosphate is lowered in the cofactor α-methylaspartate complexes. At high pH values, the chemical shift is similar to that observed in the presence of succinate. The increased line width seen for α-methylaspartate may indicate a greater tightening and restriction in the phosphate mobility, consistent with the extraordinary thermal stability of the enzyme-α-methylaspartate complexes.

An alternative explanation is that this line width may be an example of an intermediate rate of exchange between two or more distinct enzyme-α-methylaspartate complexes.

As our conception of the transaminase reaction proceeds, the initial formation of the external aldime is followed by the removal of the amino acid α-proton to form a semiquinoid intermediate (Scheme 1). The semiquinoid intermediate is then protonated at carbon 4' of the cofactor to give the ketimine which is hydrolyzed to products. erythro-β-Hydroxy-L-aspartate reacts rapidly with the enzyme, forming an equilibrium mixture of reaction intermediates. The absorption spectrum indicates the presence of two major components: the semiquinoid intermediate with an absorbance at 500 nm (ε = 20,000 cm⁻¹ M⁻¹) and the ketimine (the chemical shift is absorbance at 335 nm (ε = 8,000 cm⁻¹ M⁻¹)). The observed intensities of these two absorption bands indicates formation of about twice as much of the semiquinoid intermediate as of the ketimine. The affinity of erythro-β-hydroxy-L-aspartate and the distribution of complexes it forms is unaffected by pH (30). The two peaks observed in the 31P NMR spectra are consistent with the presence of multiple enzyme-erythro-β-hydroxy-L-aspartate complexes. The chemical shift of the upfield peak and its apparently narrower line width are somewhat similar to those parameters in the enzyme-succinate complex. The chemical shift and apparently broader line width of the downfield peak resemble that observed for the α-methylaspartate complexes. These similarities suggest that the downfield peak might arise from the ketimine, and the upfield peak may result from the semiquinoid intermediate. Further study is necessary to confirm these correlations.

The final stages of a transamination half-reaction produce the initial formation of the external aldime (Scheme 1, left to right sequence) keto acid and the pyridoxamine-P form of the enzyme. The chemical shift of the phosphate of the aminic cofactor is invariant with pH from pH 5–9 and that the observed chemical shift is similar to that proposed for monoanionic form of the aldimeylic enzyme is surprising. This stabilization could arise from a conformational change which traps the monoanionic phosphate in its binding site and physically separates it from interaction with the solvent. However, no changes in the rate of chemical modification for the aminic form of the enzyme (26) have been observed, thus making such a conformational change an unlikely interpretation. A simpler model involves a subtle conformational change in which the phosphate undergoes an electrostatic interaction such that its O—P—O bond angle is distorted to the presumed monoanionic form of the internal aldime and is maintained in this conformation throughout the complete pH range studied. Several candidates for a hydrogen-bonding donor stabilizing the distorted O—P—O angle are suggested from crystallographic studies (18). Arg-266, Ser-107, and Ser-255 could serve as hydrogen-bonding donors. Also, the freed Lys-258 and even the C-4' amino group of the cofactor are possible candidates. In addition to the polar residues at the phosphate-binding site, an α-helical segment comprised of residues 108–124 provides a positive dipolar field stabilizing the negatively charged phosphate (18). A sufficiently strong interaction between the positive dipole and a negatively charged dimionic form of the cofactor phosphate may prevent protonation of the phosphate and may induce sufficient O—P—O bond angle distortion to produce the observed chemical shift.

The sodium borohydride reduction of the internal aldime produces a sp² carbon at the 4'-position of the cofactor. This may represent an analogue of the transition state in the transaldimination phase of catalysis; however, the 31P NMR effect of reduction on the cofactor phosphate is dissimilar to effects observed for any of the enzyme forms involved in catalysis. The electronic spectrum of the internal aldime indicates conjugation of the azomethine bond with the pyridine ring of the cofactor. Maximum π-bond overlap implies coplanarity of the imine double bond and the ring, imparting a sidedness to the internal aldime. Reduction of the internal aldime with sodium borohydride, hydrolysis, an oxidative degradation of the resulting pyridoxyllysine yields pyridoxine with tritium in the re configuration at C-4' (Scheme 1, below plane of pyridine) (31). Crystallographic evidence has also indicated solvent exposure of the re face of the internal aldime (18, 19). Presumably, the initial substrate attack also occurs from the re face. Many observations have supported a transamination mechanism which requires that the substrate C-4'-proton bond (to be broken, it must be perpendicular to the pyridine ring (23). Dunathan et al. (32) specifically found the C-4' proton to be on the si or opposite to the solvent side for the cytoplasmic enzyme. A limited rotation of the cofactor ring about the C-5—C-5' bond as well as rotation of the substrate about the C-4—C-4' bond can provide the necessary flexibility to orient the si face (Scheme 1, above pyridine ring) of the substrate-cofactor complex near the catalytic groups responsible for C-4 and C-4' proton abstraction and addition. The possibility of movement of the C-5' group is consistent with the 31P NMR information we have.

Fourier difference maps of the pyridoxime-P form of the chicken heart mitochondrial enzyme show that the cofactor ring moves forward, away from the protein wall, about an axis approximately coplanar with and in the lower third of the ring (18). This is consistent with the rotation postulated through the C-5—C-5' bond. NaBH₄-treated enzyme also revealed a reorientation of the cofactor. These crystallographic studies of the aldime, or apotransaminase from chicken cannot fully provide an explanation for the NMR observation of the pig transaminase, yet they are consistent with our interpretation for a rearrangement of the phosphate environment and the O—P—O bond angles. It would be of interest to ascertain whether the conformational change in the substrate cofactor occurs concurrent with a gross protein conformational change, whether the rotation occurs at a specific step in the catalytic process, or whether either conformational change occurs as a discrete process. It is, however, of interest that those enzyme forms which are known from x-ray diffraction studies to have undergone the reorientation of the cofactor show the greatest change in the pH dependence of their 31P NMR. The postulated C-5—C-5' rotation poses a question as to the orientation of the phosphate group with respect to the pyridine ring when the cofactor is enzyme bound. Unrestricted rotation about all the bonds comprising the phosphate ester group, i.e. C-5—C-5', C-5'-O, and O—P, is unlikely because of the large volume required to accommodate such mobility. This restricted rotation is consistent with the observed 31P line width.

The 31P resonance seen for the apoenzyme of the mitochondrial enzyme (Fig. 3) indicates that this isozyme, like the apocytoplasmic isozyme (4), tightly binds inorganic phos-
have many polar neighbors poised about it in what is certainly a complicated network of changing interactions. This emphasizes the need for caution in extrapolating possible apparent homologies in the low resolution structures of the isozymes to the fine detail necessary for an understanding of specific atomic interactions related to enzyme function. These results also contribute to the diversity of the behavior of the phosphate of pyridoxal-P utilized in covalent catalysis. It seems increasingly unlikely that the phosphates of the cofactor has identical environments or, possibly, functions among pyridoxal-P-dependent enzymes. This study provides a correlation of findings in solution surroundings to those reported in crystals for a critical component of the coenzyme and points out the apparently dynamic state of this environment throughout the interconversions of enzyme forms and enzyme-substrate complexes of aspartate aminotransferase.

REFERENCES

20. Deleted in proof