1981

Aspartate aminotransferase reconstituted with N- and O-methylated vitamin B6 phosphates

Victor John Chen
Iowa State University

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CHEN, VICTOR JOHN

ASPARTATE AMINOTRANSFERASE RECONSTITUTED WITH N- AND O-
METHYLATED VITAMIN-B6 PHOSPHATES

Iowa State University

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Aspartate aminotransferase reconstituted with N- and O-methylated vitamin B₆ phosphates

by

Victor John Chen

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Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1981
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### ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AAT</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>Eapo</td>
<td>apo form of aspartate aminotransferase</td>
</tr>
<tr>
<td>E.N-MePLP</td>
<td>aspartate aminotransferase reconstituted with N-methylpyridoxal 5'-phosphate</td>
</tr>
<tr>
<td>E.O-MePLP</td>
<td>aspartate aminotransferase reconstituted with O-methylpyridoxal 5'-phosphate</td>
</tr>
<tr>
<td>kg</td>
<td>α-ketoglutarate</td>
</tr>
<tr>
<td>MDH</td>
<td>malic dehydrogenase</td>
</tr>
<tr>
<td>NADH</td>
<td>the reduced form of β-nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>N-MePLP</td>
<td>N-methylpyridoxal 5'-phosphate</td>
</tr>
<tr>
<td>N-MePMP</td>
<td>O-methylpyridoxamine 5'-phosphate</td>
</tr>
<tr>
<td>mmr</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>ORD</td>
<td>optical rotary dispersion</td>
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<td>O-MePLP</td>
<td>O-methylpyridoxal 5'-phosphate</td>
</tr>
<tr>
<td>O-MePMP</td>
<td>O-methylpyridoxamine 5'-phosphate</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal 5'-phosphate</td>
</tr>
<tr>
<td>PMP</td>
<td>pyridoxamine 5'-phosphate</td>
</tr>
<tr>
<td>TEA.HCl</td>
<td>triethanolamine hydrochloride</td>
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INTRODUCTION

Aspartate aminotransferase (EC 2.6.1.1) is an enzyme that catalyzes the reversible transfer of an amino group from L-aspartate to α-ketoglutarate as shown in equation (1):

\[ \text{L-aspartate + α-ketoglutarate} \rightleftharpoons \text{oxalacetate + L-glutamate} \]  

(1)

This reaction is known to play a critical role in nitrogen metabolism in the cell. At the same time, aspartate aminotransferase, which exists as mitochondrial and cytoplasmic isoenzymes, is believed to be involved in the malate-aspartate shuttle whereby reducing equivalents enter the mitochondria from the cytoplasm (Dawson, 1979). The activity of the enzyme is completely dependent on the presence of the 5'-phosphorylated form of vitamin B₆ which serves as the coenzyme in the active site. The enzyme is a key vitamin B₆-binding protein in the cell. Some 30% of the pool of protein-bound vitamin B₆ in the cytoplasm and as much as 90% in the mitochondria are found in the active sites of molecules of aspartate aminotransferase (Borson et al., 1978). To clinicians, this enzyme is better known as glutamate-oxalacetate transaminase. Its serum activity has been used for many years as an index of heart and liver disease (Kishino et al., 1968). The physiological, nutritional and clinical relevance of this enzyme lend importance to the understanding of its structure and chemistry in solution.

Many aspects of the reaction catalyzed by aspartate aminotransferase make it an excellent candidate for enzymological studies. Its vitamin B₆-containing coenzyme, pyridoxal 5'-phosphate (PLP), by itself can catalyze the reaction of equation (1) in the absence of the protein
albeit at much reduced efficiency. The mechanisms of the nonenzymatic reaction have been elucidated (Metzler et al., 1954; Metzler, 1977). The existence of such a congruent nonenzymatic model allows enzymologists to isolate the contribution of the protein towards catalysis of the chemical reaction.

In this work, my interest is focused on the interaction between the coenzyme PLP and the protein matrix during catalysis. The coenzyme can be considered as part of the active site of the enzyme. Knowledge of the interaction of PLP with the protein matrix is crucial for an understanding of the catalytic mechanism of aspartate aminotransferase.

Pyridoxal 5'-phosphate is a chromophore whose electronic absorption spectrum has been extensively investigated (Harris et al., 1976). Bound at the active site, the optical properties of PLP are very sensitive to changes in the chemical structure of the protein. Thus, the coenzyme functions as a reporter group buried in the protein. Observed changes in the spectrum reflect alterations at the active site at different stages of enzymatic processes.
Another attractive aspect of aspartate aminotransferase with respect to the study of coenzyme-protein interaction is the fact that the vitamin B₆ cofactor can be removed readily from the protein. In its place, a selection of analogs of pyridoxal 5'-phosphate with various structural modifications can be incorporated. The apoenzyme reconstituted with these analogs displays different degrees of activity. The study of these apoenzyme-analog complexes can provide clues to the mechanisms of interaction between the coenzyme and various functional groups on the protein.

Recently, the crystallization of both the mitochondrial and the cytoplasmic isoenzymes has sparked new excitement in the enzymology of transamination (Gehring et al., 1977; Arnone et al., 1977; Borisov et al., 1978). The three-dimensional structures of these proteins are now being determined by X-ray crystallography. The crystalline enzymes have been shown to be catalytically competent. Crystalline enzyme-substrate complexes of natural substrates and various inhibitory ligands as well as crystalline apoenzyme-analog complexes have been prepared (Metzler et al., 1978). Many of these possess linear dichroism and their polarized light absorption spectra have been recorded. Observations of these spectra together with the forthcoming knowledge of the three-dimensional structures of the proteins will provide a means of examining the interaction of the coenzyme with the protein at the atomic level.

The study reported in this thesis was undertaken to further characterize two apoenzyme-analog complexes of aspartate aminotransferase. The N- and O-methylated derivatives of pyridoxal 5'-phosphate have been incorporated into the enzyme (Furbish et al., 1969; Mora et al., 1972).
Although they possess no measurable catalytic activity, the reconstituted enzymes apparently can bind and react with substrate. It is the intent of this work to find out how methylation of the coenzyme leads to inactivity.
LITERATURE REVIEW

The structure of aspartate aminotransferase

The cytoplasmic isoenzyme of aspartate aminotransferase (AAT) from porcine heart was first isolated by Jenkins et al. (1959). Later, it was shown that this preparation was a mixture of several subforms which were separable by starch gel electrophoresis (Martinez-Carrion et al., 1967). Three major bands were seen and they were designated α, β, and γ, in order of increasing mobility towards the anode.

The subforms appear to be very similar in molecular weight, amino acid sequence and immunoprecipitation behavior. However, they do differ in activity. α is the most active; relatively, β is a little more than half and γ is less than a third as active.

The α subform is the best studied. This enzyme is a dimeric protein with two identical subunits. Each subunit is composed of 412 amino acids whose primary sequence has been determined (Ovchinnikov et al., 1973; Doonan et al., 1975). The molecular weight of each subunit is calculated to be 46344. One molecule of vitamin B₆ is covalently bound to each subunit via a Schiff base linkage to the ε-amino group of Lys-258 (Hughes et al., 1962) as shown in Figure 1.

The crystals of cytoplasmic AAT are grown by vapor diffusion from solutions of polyethylene glycol. X-ray diffraction patterns show that they belong to the orthorhombic space group P2₁2₁2₁ with unit cell dimensions a = 124.7 Å, b = 130.9 Å, and c = 55.7 Å (Arnone et al., 1977).

In the 2.7 Å electron density map (Arnone, private comm., 1981), the coenzyme can be seen clearly lying against the side of a β sheet. A
Figure 1. The active site of aspartate aminotransferase  
(Courtesy of Mr. C. Craig Hyde)
nearly identical structure exists for the mitochondrial isoenzyme (Ford et al., 1980). The phosphate group is bonded to a number of functional groups from the protein. These include the guanidinium group of one arginine and hydroxyl groups from a serine, a threonine and a tyrosine residue. There also appears to be interactions between the phosphate group and one or more amide linkages at the N-terminus of a long helix.

The coenzyme ring is at the end of the extended side chain of Lys-258. Both the phenolic oxygen and the imine nitrogen of the coenzyme are bonded to phenolic groups of tyrosine residues, one of which (Tyr-225) protrudes from the β sheet. The imine double bond assumes a syn conformation with respect to the phenolic oxygen of the coenzyme. The ring nitrogen of the coenzyme is hydrogen bonded with an aspartate residue (Asp-222).

There is no histidine residue immediately within bonding distances of the coenzyme. The closest are two histidine residues some 5-7 Å below the coenzyme and buried in the protein.

The crystallographic results are supplemented by studies on the mode of binding of the coenzyme in solution by Phosphorus-31 nmr (Martinez-Carrion, 1975). The phosphate group is bound in its dianionic form. It cannot be titrated and remains in this form even upon binding of substrates or other inhibitory ligands to the active site.

The pK for dissociation of a proton from the bound coenzyme

The nitrogen of the imine linkage can be protonated reversibly. The protonated and deprotonated forms absorb at 430 and 362 nm, respectively. Both absorption bands exhibit positive circular dichroism (CD). The
observed pK (pK_o) has been determined spectrophotometrically as 6.3 at ionic strength 0.1 (Jenkins & Sizer, 1959; Fonda & Johnson, 1970).

The presence of anions in the buffer has extraordinary effects on the value of pK_o. A buffered solution of the enzyme at pH 6.3 is bright yellow. Upon deionization the solution turns colorless. The yellow color reappears when chloride ions are added. For the deionized enzyme, the value of pK_o has been estimated as 5.3 ± 0.1 (Bergami et al., 1968; Giannini et al., 1975). As the anion concentration increases, the value of pK_o gradually increases to a limiting value that depends upon the nature of the anion. The maximum attained for monovalent anions is about 7 (Jenkins, 1980). For a few dicarboxylates such as succinate, glutarate, oxalacetate and α-ketoglutarate, the value of pK_o can plateau as high as 9 (Jenkins & D'Ari, 1966a; Fonda & Johnson, 1970). The wide variation in the value of pK_o is believed to result from binding of anions to specific sites on the protein rather than simply being caused by a change in the ionic strength of the medium. Therefore, the plateau value of pK_o is that of the protonated imine of the enzyme-anion complex.

The nature of anion binding

Depending on the values of pK_o of their complexes with the aldime enzyme, dicarboxylates can be divided into two classes. Class A compounds, which include the straight chain dicarboxylates mentioned above, form enzyme-anion complexes with the values of pK_o at about 9. Class B compounds behave very similarly to monovalent anions and form enzyme-anion complexes with the values of pK_o only at about 7 (Bonsib et al., 1975).
A model has been suggested (Jenkins & D'Ari, 1966a) for the binding of anion compounds to the aldimine form of AAT. It was proposed that there are two independent positively charged residues A and B on the enzyme. One of these, A, is thought to interact with the bound imine. Binding of an anion to A results in alteration of the value of pK_o of the imine group of the coenzyme. Moreover, the orientation of A with respect to B is such that only an inhibitor of Class A is structurally capable to bind to both sites simultaneously. Monovalent anions such as halides and acetate can individually bind to both A and B. However, the aromatic dicarboxylates of Class B cannot bind to A. They can only bind to B.

Two pieces of evidence are consistent with this model. One is the observation that the affinity of AAT for a dicarboxylate increases as the pH is lowered (Cheng & Martinez-Carrion, 1972). The other is that at pH 8, one monovalent anion competes with dicarboxylates for the binding to AAT while at lower pH, more than one monovalent anion is involved (Cheng et al., 1971; Cheng & Martinez-Carrion, 1972; Jenkins & D'Ari, 1966a). These are explicable in terms of the above model. At pH 8, only residue B can bind a ligand. Because the nearby imine group of the bound coenzyme is unprotonated at this pH, residue A has little affinity for an anion. However, as the pH is lowered, the imine becomes protonated, and residue A is available for anion binding as well. Thus, the stoichiometry of anion competition with the binding of a dicarboxylate depends on the number of monovalent anions that can be bound at a given pH.
Fluoroacetate has been used to probe the nature of the anion-binding site in AAT using $^{19}$F-nmr (Cheng & Martinez-Carrion, 1972). It was found that fluoroacetate can bind not only to the aldimine form of AAT, it can also bind to the apo form, the amine form and to the NaBH$_4$-reduced aldimine form of the enzyme. The interesting thing is that in each case, the binding site involves an ionizable residue of the protein with an apparent pK of 6.2 ± 0.2. Unfortunately, data below pH 6 become unreliable and no information can be obtained regarding whether a second site is involved at lower pH.

The exact identity of the group with pK 6.2 is uncertain. It is interesting that photooxidation of one to two histidine residues results in the complete loss of anion effects on the binding of $\alpha$-methylaspartate and of glutarate (Peterson & Martinez-Carrion, 1970). Although the modified enzyme can only undergo transamination slowly, no significant damage to its overall structure is evident as judged by its ORD spectrum and immunoprecipitation behavior (Martinez-Carrion et al., 1970). However, as pointed out before, in the electron density map, no histidine is seen in the active site. The likelihood that either residue A or B, alluded to in the anion-binding model, is histidine seems remote.

By chemical modification experiment, arginine has been implicated as a candidate for one of the anion binding sites (Azaryan et al., 1978; Gilbert & O'Leary, 1975; Riordan & Scandurra, 1975). Usually more than one arginine residue is modified in these experiments, and it was not possible to identify the locations of the anion-binding residues.
However, from the electron density maps it seems clear that these are Arg-386 (group A) and Arg-292 (group B) of the second subunit (Ford et al., 1980; Arnone, private communication, 1981).

Because so many different kinds of anions can bind to the enzyme, the binding of ligands to AAT can be viewed as an ion-exchange process depicted below:

**Scheme 1**

\[
\text{Enzyme.anion + ligand} \rightarrow \text{Enzyme.ligand + anion}
\]

Indeed, when the concentration of the anion in solution is extrapolated to zero, the dissociation constant for glutarate is found to approach zero (Jenkins & D'Ali, 1966a). Therefore, the variation in affinity of the enzyme for any ligand can be a result of anion binding.

**The effects of anion on substrate binding**

The transamination reaction catalyzed by AAT is known to be sensitive to the type and concentration of buffer ions (Boyde, 1968; Nisselbaum, 1968). The ion-exchange mechanism of Scheme 1 also applies to substrate binding. In view of this, it has been postulated that the *in vivo* activity of AAT is regulated by the intracellular anion concentrations (Harruff & Jenkins, 1976).

Dicarboxylates of both Class A and B are inhibitory to substrate binding. Despite the structural similarity between substrate amino acids and dicarboxylates, their modes of binding may be different. While the stoichiometry for monovalent anion competition with the binding of a dicarboxylate increases with decreasing pH, that with the binding of the substrate analog, *erythro-*β-hydroxy-L-aspartate, is
invariant in the pH range of 6 to 9 (Harruff & Jenkins, 1978). Furthermore, both the equilibria between all the anzyme-substrate intermediary complexes as well as the maximal velocity of the enzymatic activity are insensitive to the change in pH (Cheng et al., 1971; Harruff & Jenkins, 1978; Jenkins, 1964; Jenkins & D'Aré, 1966b; Jenkins & Taylor, 1965; Velick & Vavra, 1962).

**The mechanism of transamination**

Independently, Braunstein & Shemyakin (1953) and Metzler et al., (1954) proposed a bioorganic mechanism for the reversible transamination of an amino acid as catalyzed by pyridoxal 5'-phosphate. On the basis of this mechanism, together with studies of light absorption and of circular dichroism (CD), a mechanism of transamination has been advanced that includes the interaction of the prosthetic group with the protein matrix during catalysis (Ivanov & Karpeisky, 1969).

The role of the protein is presumably to orient the substrate and the coenzyme correctly at each stage of the catalytic reaction. Upon binding of substrates, changes of CD are observed in the spectral regions of both the coenzyme and of the aromatic residues of the protein (Ivanov et al., 1967). These changes were interpreted by Ivanov and Karpeisky as due to the rotation of the coenzyme ring with a concomitant conformational alteration of certain aromatic residue during catalysis.

According to the mechanism of Ivanov and Karpeisky (Scheme 2), the first step is the binding of an amino acid in the zwitterionic form to the enzyme with a deprotonated aldimine to form a Michaelis complex as in structure I in Scheme 2. By analogy with the binding of succinate...
or glutarate, there is an increase in the value of $pK_o$ for the bound prosthetic group. The result is that the $pK_o$ of the imine group is comparable to that of the amino group of the substrate, both being about 9. Proton transfer can occur from the amino group to the imine linkage. Protonation of the imine group was proposed to decrease the charge density around the 4'-carbon of the coenzyme and to facilitate nucleophilic addition there in step 2 to form the tetrahedral adduct III.

In step 3, proton transfer is again proposed to take place between the geminal amino group leading to the elimination of the lysyl side chain in step 4. Steps 2 to 4 are referred to as transimination. The coenzyme forms a protonated Schiff base with the substrate amino acid in V. Complex V displays an absorption band at 430 nm, but there is no CD.

Subsequent abstraction of the proton on the $\alpha$-carbon of the substrate in step 5 generates the quinonoid intermediate VI. This intermediate is characterized by a tall and slender band at 492 nm and a small negative CD. Reprotonation at the 4'-carbon of the coenzyme in step 6 converts VI into a ketimine VII. Upon hydrolysis in step 7, the product keto acid is liberated. The amine form of the coenzyme is left bound in the active site. It has an absorption band centered at 330 nm and displays positive CD.

**Enzyme-substrate complexes**

Depending on the substrate, one or more, but not all, of the intermediates in Scheme 2 can be observed spectrophotometrically at equilibrium. When aspartate or glutamate is added to the enzyme at pH 8,
the absorption band at 362 nm due to the free enzyme immediately decreases in intensity while bands appear at 490, 430 and 330 nm (Jenkins & D'Ari, 1966b; Fonda & Johnson, 1970). The associated CD bands are negative, zero and positive respectively (Ivanov et al., 1967). These spectral maxima correspond to enzyme-substrate intermediary complexes VI, V and a mixture of VII and VIII.

Erythro-β-hydroxy-L-aspartate reacts with the enzyme at pH 8 to produce a similar mixture of complexes but containing a large amount of VI (Jenkins, 1961).

With α-methylaspartate, at pH 8, complex V and I can be seen. This substrate analog can react only through the transimination step. The reaction cannot proceed further because the α-proton has been substituted by a methyl group (Jenkins et al., 1959).

Inasmuch as binding of substrates is followed immediately by transamination, the trapping of complex I alone is virtually impossible. However, the enzyme-dicarboxylate complexes may be analogous to the Michaelis complex I. The absorption band of the aldimine enzyme-dicarboxylate complex is at 430 nm and exhibits a positive CD (Ivanov et al., 1967).

Reaction of L-cycloserine with AAT produces a complex analogous to VIII in Scheme 2. It has an absorption band at 330 nm and displays positive CD (Ivanov et al., 1967).

Aspartate aminotransferase reconstituted with vitamin B₆ analogs

The interaction of vitamin B₆ analogs with the apo form of aspartate aminotransferase and of other enzymes has been the subject of several reviews (Snell 1958, 1970; Braunstein 1973). A summary of results
obtained with most of the analogs with a 4-aldehyde substituent that have been tested with aspartate aminotransferase are collected in Table 1. Results for other analogs are collected in Table 2. Since the binding of the aldehyde form of an analog is nearly irreversible, the equilibrium constant for the binding process cannot be established easily. In Table 1, the constants $K_{\text{co}}$ and $K_i$ are analogous to the usual dissociation and inhibition constants but are not true equilibrium constants (Fonda, 1971; Hullar, 1969; Meister et al., 1954; Morino & Snell, 1967; O'Leary, 1969). It has been well recognized that the binding of vitamin B₆ analogs is very sensitive to the substituent at the 5'-position of the coenzyme (Evangelopoulos & Sizer, 1965; Wada & Snell, 1962; Snell 1970). Comparison between the available values of $K_{\text{co}}$ or $K_i$, those analogs lacking a negatively charged 5'-substituent, such as compounds 21 and 22, have their values of $K_{\text{co}}$ in the mM range while those possessing a doubly charged 5'-substituent have theirs at the μM range or below. The analogs with a singly charged 5'-substituent, such as 8 and 9 have constants intermediate between the above (Fonda, 1971). Modification at other positions of the coenzyme has little effect on binding (Snell, 1970). The values of $K_{\text{co}}$ or $K_i$ for compounds 3 and those in Table 2 are at the μM range or below (Fonda, 1971; Hullar, 1969). In fact, the dissociation constant for the binding of inorganic phosphate is also in the μM range (Verge et al., 1979).

In spite of their high affinity for the active site of the apoenzyme, only a few compounds display more than half of the coenzyme activity of pyridoxal 5'-phosphate. Modification at the 2- and 6-positions of the
Table 1. Properties of vitamin B₆ coenzyme analogs with a 4'-aldehyde

<table>
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<th>Compounds</th>
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<th>2</th>
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<tr>
<td>(K_{\text{CO}}) (µM)</td>
<td>0.15¹,0.01⁵</td>
<td>0.07¹</td>
<td>1.5²</td>
</tr>
<tr>
<td>(K_1) (µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>binding time (%1:1) ratio, pH 8.3</td>
<td>&lt;2 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\lambda_{\text{max}}) of bound analog (nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\sim \text{pH 5.2} )</td>
<td>382</td>
<td>425¹</td>
<td>365¹</td>
</tr>
<tr>
<td>(\sim \text{pH 8.2} )</td>
<td>430</td>
<td>360¹</td>
<td></td>
</tr>
<tr>
<td>(\Delta A/A_0 \times 10^4) (peak position nm)</td>
<td>20⁴(360)</td>
<td>21¹(260)</td>
<td>--</td>
</tr>
<tr>
<td>(pK_O)</td>
<td>6.3</td>
<td>5.8¹</td>
<td>6.5¹</td>
</tr>
<tr>
<td>relative activity %</td>
<td>100</td>
<td>120¹,180²</td>
<td>322</td>
</tr>
<tr>
<td>(K_m) (for substrate, mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with (L)-glutamate°¹</td>
<td>&lt;2 min⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with (\alpha)-ketoglutarate°¹</td>
<td></td>
<td>&lt;2 min⁴</td>
<td></td>
</tr>
<tr>
<td>reaction with (\alpha)-methylaspartate°¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with (\text{})erythro-3-hydroxyaspartate°¹</td>
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°¹\(L\)-glutamate at \(\sim 18\) mM, pH 8.3.

°²\(\alpha\)-ketoglutarate, final concentration \(\sim 8\) mM, added after glutamate reaction is completed, pH of mixture 6.5.

°³\(18\) mM of DL-mixture, pH 8.3.

°⁴5±2 mM, pH 8.
<table>
<thead>
<tr>
<th>Compounds</th>
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<th>7'</th>
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<td>370(^3)</td>
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<tr>
<td>15(^1) (360)</td>
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<td>6.4(^1)</td>
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<td>6.4(^3)</td>
<td>7.1(^3)</td>
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<td>50</td>
<td>56(^1)</td>
<td>47(^1)</td>
<td>90(^3)</td>
<td>&lt;0.2(^4)</td>
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<td>0.08(^1) (α-KG)</td>
<td>0.14(^1) (α-KG)</td>
<td>1.0(^3) (α-KG)</td>
<td>1.0(^3) (L-Asp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6(^1) (L-Asp)</td>
<td>1.0(^1) (L-Asp)</td>
<td>10(^3) (L-Asp)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<2 min, gives 319 nm, no CD\(^6\), slow\(^4\)

small shoulder at 430 nm with negative CD\(^6\)

490 band with negative CD seen at once, it decays with half life of 4 min\(^6\)
<table>
<thead>
<tr>
<th>Compounds</th>
<th>2</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_C$ (μM)</td>
<td>270$^5$</td>
<td></td>
</tr>
<tr>
<td>$K_i$ (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>binding time ν:1:1 ratio, pH 8.3</td>
<td>2 min$^4$</td>
<td>ν40 min$^6$</td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$ of bound analog (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>νpH 5.2</td>
<td>370$^4$</td>
<td></td>
</tr>
<tr>
<td>νpH 8.2</td>
<td>370$^4$</td>
<td>365,417$^6$</td>
</tr>
<tr>
<td>$\Delta A/Ax10^4$ (peak position nm)</td>
<td>15$^4$(365)</td>
<td>10$^6$</td>
</tr>
<tr>
<td>$pK_o$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>relative activity %</td>
<td>&lt;0.2$^4$</td>
<td></td>
</tr>
<tr>
<td>$K_m$ (for substrate, mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with L-glutamate$^b$</td>
<td>80% in 2 min to give band at 316 nm$^4$</td>
<td>biphasic reaction, &lt;1 min to give 318 nm, No CD$^6$</td>
</tr>
<tr>
<td>reaction with α-ketoglutarate$^c$</td>
<td>v. slow$^4$</td>
<td></td>
</tr>
<tr>
<td>reaction with α-methylaspartate$^d$</td>
<td></td>
<td>no change$^6$</td>
</tr>
<tr>
<td>reaction with erythro-β-hydroxyaspartate$^e$</td>
<td></td>
<td>490 nm band seen at once, it decays with half life of 5 min$^6$</td>
</tr>
<tr>
<td>Compounds</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>-----------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>8.8⁵</td>
<td>4.1⁵</td>
<td></td>
</tr>
<tr>
<td>2 min⁴</td>
<td>&lt;2 min⁴</td>
<td>&lt;2 min⁶</td>
</tr>
<tr>
<td>431⁴</td>
<td>425⁴</td>
<td>375⁴</td>
</tr>
<tr>
<td>335,339,420⁴</td>
<td>375⁴</td>
<td></td>
</tr>
<tr>
<td>10⁴(355)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>&lt;0.2⁴</td>
<td>3⁴</td>
<td></td>
</tr>
</tbody>
</table>

- slow to give band at 326 nm, 430 nm transient seen. No CD for either⁴,⁶
- v. slow⁴ <2 min to give band at 427 nm⁴
gives 430 nm band with negative CD⁶
- gives 430 nm band with negative CD⁶
- 490 nm seen at once, no CD at 300 nm⁶
- 490 decays with half life of 10 min⁶
  488 nm band very similar intensity to native enzyme⁸
<table>
<thead>
<tr>
<th>Compounds</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_C$ ($\mu$M)</td>
<td>0.54$^7$</td>
<td>~15 min$^6$</td>
</tr>
<tr>
<td>$K_d$ ($\mu$M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>binding time 1:1 ratio, pH 8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\lambda_{max}$ of bound analog (nm)</td>
<td>3646</td>
<td></td>
</tr>
<tr>
<td>$\sim$pH 5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\sim$pH 8.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta A/Ax10^4$ (peak position nm)</td>
<td>18(365)$^8$</td>
<td>2.2(363)$^6$</td>
</tr>
<tr>
<td>$pK_o$</td>
<td>$\sim$5$^10$</td>
<td></td>
</tr>
<tr>
<td>relative activity %</td>
<td>27,15$^8$</td>
<td></td>
</tr>
<tr>
<td>$K_m$ (for substrate, mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with L-glutamate</td>
<td>give band at 340 nm with positive CD$^8$</td>
<td>biphasic reaction, 2 min to give 314, no CD$^6$</td>
</tr>
<tr>
<td>reaction with $\alpha$-ketoglutarate$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with $\alpha$-methylaspartate$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with erythro-$\beta$-hydroxyaspartate$^e$</td>
<td>gives 498 band very similar intensity to native enzyme$^8$</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Reaction with $\alpha$-ketoglutarate gives 498 band very similar intensity to native enzyme.

$^2$Reaction with L-glutamate gives band at 340 nm with positive CD.

$^3$Biphasic reaction, 2 min to give 314, no CD.

$^4$Reaction with $\alpha$-methylaspartate.

$^5$Reaction with erythro-$\beta$-hydroxyaspartate.

$^6$Relative activity.

$^7$Kd value.

$^8$pKo value.
<table>
<thead>
<tr>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>~30 min$^6$</th>
<th>~25 min$^6$</th>
<th>&gt;20 min$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>365,426$^6$</td>
<td>379,435$^6$</td>
<td>369$^4$</td>
</tr>
<tr>
<td>18(363)$^4$</td>
<td>6(366)$^6$</td>
<td>13(367)$^4$</td>
</tr>
</tbody>
</table>

increases with time$^4$

biphasic reaction, 1 min to give 314 nm no CD$^6$
biphasic reaction in 260 min gives 314,413 nm with no CD$^6$
slow to give 317 nm$^4$
slow$^4$

no change$^6$
no change$^6$

no peak at 490 nm. instead gives 314 nm. no CD$^6$ 490 nm band seen immediately$^6$, it decays with half life of 8 min
Table 1. Continued

<table>
<thead>
<tr>
<th>Compounds</th>
<th>21</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_c^0$ (μM)</td>
<td>&gt;500$^5$</td>
<td>loosely bound$^6$</td>
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<tr>
<td>$K_d$ (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>binding time ~1:1 ratio, pH 8.3</td>
<td>slow</td>
<td></td>
</tr>
<tr>
<td>$\lambda_{\text{max}}$ of bound analog (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\nu$ pH 5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\nu$ pH 8.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta A/Ax10^4$ (peak position nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$pK_o$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>relative activity %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (for substrate, mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with L-glutamate$^b$</td>
<td>&lt;2 min for most part to give 321 nm, 430 nm decreases slowly$^4$</td>
<td>complete reaction in 20 min$^6$</td>
</tr>
<tr>
<td>reaction with $\alpha$-ketoglutarate$^c$</td>
<td>&lt;2 min partially$^4$</td>
<td></td>
</tr>
<tr>
<td>reaction with $\alpha$-methylaspartate$^d$</td>
<td></td>
<td>430 nm band seen, with small negative CD$^6$</td>
</tr>
<tr>
<td>reaction with erythro-$\beta$-hydroxyaspartate$^e$</td>
<td></td>
<td>490 nm band seen immediately, no CD seen$^6$</td>
</tr>
<tr>
<td>Compounds</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>0.5&lt;sup&gt;9&lt;/sup&gt;</td>
<td></td>
<td>4.45</td>
</tr>
<tr>
<td>&lt;2 min&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 min&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>322,382,408&lt;sup&gt;4&lt;/sup&gt;</td>
<td>380,420&lt;sup&gt;4&lt;/sup&gt;</td>
<td>310(shoulder)&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>392&lt;sup&gt;4&lt;/sup&gt;</td>
<td>377&lt;sup&gt;4&lt;/sup&gt;</td>
<td>310(shoulder)&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>positive at 380&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5&lt;sup&gt;11&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 min to give band at 326 nm&lt;sup&gt;4&lt;/sup&gt;</td>
<td>slow to give at 335 nm&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5 min to give a small transient at 485 nm&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;2 min&lt;sup&gt;4&lt;/sup&gt;</td>
<td>v. slow&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5 min, loss of 485 nm&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
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<td></td>
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</table>
Table 1. Continued

<table>
<thead>
<tr>
<th>Compounds</th>
<th>27</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{c0}$ (μM)</td>
<td>covalently modified</td>
<td>covalently modified</td>
</tr>
<tr>
<td>$K_d$ (μM)</td>
<td>AAT$^{12}$</td>
<td>AAT$^{13}$</td>
</tr>
<tr>
<td>binding time $\approx 1:1$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ratio, pH 8.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\lambda_{max}$ of bound analog (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\approx$ pH 5.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\approx$ pH 8.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta A/Ax10^4$ (peak position nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$pK_o$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>relative activity %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (for substrate, mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with L-glutamate$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with $\alpha$-ketoglutarate$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with $\alpha$-methylaspartate$^d$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction with 2-erythro-$\beta$-hydroxyaspartate$^e$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Properties of other vitamin B$_6$ analogs

<table>
<thead>
<tr>
<th>Compounds</th>
<th>29</th>
<th>30</th>
<th>31</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki (M)</td>
<td>0.7$^a$</td>
<td>1.7$^a$</td>
<td>1.0$^a$</td>
<td>1.4$^a$</td>
</tr>
</tbody>
</table>

$^a$Hullar, 1969.
coenzyme, such as compounds 2 to 7, does not affect the coenzyme activity a great deal (Bocharov et al., 1968; Mamaeva et al., 1971; Morino & Snell, 1967). For the enzyme reconstituted with these active analogs, the values of $K_m$ for substrates, L-aspartate and α-ketoglutarate, remain the same order of magnitude as those for the native enzyme. In view of the sensitivity of the values of $K_m$ towards the buffer system used in the assay (Boyde, 1968), the affinity of the reconstituted enzyme for substrates can be regarded as the same as that of the native enzyme.

The interaction of the natural coenzyme pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP), with the protein matrix of aspartate aminotransferase has been studied in many ways. The results from crystallographic and $^{31}$P-nmr studies of the active site have already been discussed. The coenzyme is very tightly bound. Besides the Schiff base linkage, the prosthetic group is anchored to the protein via a number of hydrogen bonds and charge interactions at N-1, the 3-phenolic and 5'-phosphate groups.

Resolution of the aldimine form of the coenzyme is impossible except under denaturing conditions. Upon conversion to the amine form, the prosthetic group can be displaced by a high concentration of either sulfate (Wada & Snell, 1962) or phosphate (Scardi et al., 1963) at pH 5. The apoenzyme can bind phosphate, apparently 2 moles per monomer (Verge et al., 1979). One of these is tightly bound and presumably occupies the same site normally filled by the coenzyme.

Reconstitution with either PLP or PMP is inhibited by divalent anions such as sulfate and phosphate (Fonda & Auerback, 1976; Severin &
Dixon, 1968). Monovalent anions also have an effect; nitrate inhibits while chloride appears to activate binding (Fonda & Auerback, 1976). Binding of the coenzyme is a multistep process and involves the dipolar ionic form of the coenzyme (Churchich, 1972; Fonda & Auerback, 1976). For the binding of PLP, as many as four steps have been detected spectrophotometrically (Arrio-Dupont, 1969). The binding of PMP is controversial: it is yet to be resolved whether anticooperativity is involved between the two subunits of the dimeric protein (Arrio-Dupont, 1972; Churchich & Farrelly, 1969; Lee & Churchich, 1975; Schlegel & Christen, 1974).

The importance of the 4-aldehyde substituent in catalytic transamination has long been recognized (Snell, 1958, 1970). Therefore, analogs lacking this group will not be expected to display any coenzyme activity. Thus, all compounds included in Table 2 have no coenzyme activity.

Modification at the 5'-position has a large effect on coenzyme activity (Furbish et al., 1969; Han, 1978). Shortening the chain length as in compound 11 resulted in complete inactivity (Furbish et al., 1969). At best, 20% coenzyme activity is obtained for compound 15 (Miura et al., 1980). Modification at N-1 or the 3-phenolic oxygen gave functionless coenzymes, compound 23 to 26 (Furbish et al., 1969).

Like the native bound coenzyme, a number of the bound aldehyde analogs are pH indicators, and their values of $pK_a$ have been determined (Mamaeva et al., 1971; Morino & Snell, 1967; Miura et al., 1980). Some of these are considerably different from the $pK_a$ value of the native
enzyme and they vary over a range of about three pH units. The lowest values are those of compounds 14 and 15 in which the 5'-bridge oxygen has been replaced (Miura et al., 1980). This phenomenon is not understood. It is suggested that in compounds 14 and 15, the low pK\textsubscript{a} values are due to an alteration in charge interaction between the 5'-phosphate group with the protein. This might be brought about by a change in the conformation of the 5'-chain or by protonation of the phosphonate group prior to protonation of the imine.

In Table 1, it is seen that most of the inactive analogs are capable of transaminating L-glutamate, i.e., compounds 8 to 12; 16 to 18 and 20 to 25 (Furbish et al., 1969; Han, 1978). The reversibility of the reaction has been demonstrated in compounds 8, 9, 11, 12, 20 and 21. However, the fact that no circular dichroism is detected for the band at 330 nm after the addition of substrate suggests that the amine form of the coenzyme analog is released from the protein (Han, 1978). If this is so, it is not surprising that these analogs possess little coenzyme activity.

The reaction of various bound inactive analogs with α-methyl-aspartate and erythro-β-hydroxy-L-aspartate give differing amounts of absorption at 430 nm and 490 nm (Han, 1978). One interpretation is that the distributions of enzyme-substrate intermediary complexes at equilibrium in the analog-reconstituted enzyme are unlike those in the native enzyme.

There are two aldehyde analogs, 27 and 28, which react very differently from the others (Yang et al., 1974; Miura & Metzler, 1976). Both compounds 27 and 28 covalently label the apo form of the enzyme.
when bound to the active site. The interesting thing is that all the spectral changes seen during the inactivation reaction can be simulated in model systems in which substituted amines are used instead of the protein. The proposed mechanisms for the nonenzymatic model reactions are shown in Schemes 3 and 4. In the enzyme, a nucleophile X is proposed to be unmasked and become activated during the process of reconstitution. It adds to the 4'-carbon of the coenzyme analog in a manner very similar to that shown in Schemes 3 and 4.

Compounds 32 to 35 were intended to be active site-directed reagents on the basis of a preceding result indicating that a Michael addition to 4-vinylpyridine by a thiol group is possible (Yang et al., 1975). Compounds 32 to 35 were shown to bind tightly to the active site of AAT. Their binding resulted in perturbation of certain aromatic residues of the protein. Lacking an aldehyde in the 4-position, none of these analogs has coenzyme activity. Compounds 32 to 34 apparently do not covalently react with the protein; they can be displaced by the native coenzyme. Compound 35 reacts with the apo form of AAT in a complex way that is reminiscent of the behavior of compounds 28 and 29. The reaction takes several hours to complete. The bound analog can be released only upon denaturation of the protein. Reduction of the final complex results in covalent attachment of compound 35 to the protein. Scheme 5 shows the proposed mechanism of reaction.

The Schiff base formed between pyridoxal 5'-phosphate and an amino acid can be reduced with NaBH₄ resulting in the amino acid portion being permanently affixed to the 4'-position of the coenzyme. Such reduced Schiff bases have been shown to bind very tightly to the active site of
Scheme 3

\[ Y \quad H_2N \quad XH \quad OH \quad \text{HSO}_3^- \quad \rightarrow \quad \text{N}^+ \quad \text{H}^+ \quad \text{OSO}_3^- \]  

\[ Y \quad H \quad \text{N} \quad XH \quad \text{OSO}_3^- \quad \rightarrow \quad \text{N}^+ \quad \text{H}^+ \quad \text{O} \quad \text{OSO}_3^- \]

\[ \text{H}^+ + \text{SO}_4^= \]

\[ Y \quad \text{N} \quad \text{H} \quad \text{OSO}_3^- \quad \rightarrow \quad \text{N}^+ \quad \text{H}^+ \quad \text{O} \quad \text{OSO}_3^- \]

\[ + \text{H}^+ \]

\[ Y = \text{CO}_2^- \]

\[ X = \text{S} \]
Scheme 4

\[
\begin{align*}
Y &= \text{CO}_2^- \\
X &= \text{N}
\end{align*}
\]
Scheme 5

$\text{NH}_2$

$\text{OPO}_3^-$

$\text{NH}_2$

$\text{OPO}_3^-$

$\text{NaBH}_4$

$\text{OPO}_3^-$
AAT. They have been exploited to introduce into the active site special functional groups for various purposes; for example, heavy atoms for the purpose of phase determination in crystallographic studies or spin labels for magnetic resonance studies. However, not all of these goals are realized because unexpected side reactions often take place.

The reduced Schiff base of glutamate binds to the active site but also undergoes a side reaction in which the phosphate group is transferred to a threonine residue of the protein (Khomutov et al., 1969; Severin & Dixon, 1968). The reduced Schiff base radicals bind in the active site and oxidize cysteine 390 in the process (Misharin et al., 1974, 1975a, 1975b, 1975c). Interestingly, radicals can also bind to the aldimine form of the holoenzyme and compete with the binding of erythro-β-hydroxyaspartate. This suggests that reduced Schiff bases of amino acids are bound to both the substrate and the coenzyme sites.

Compound is intended to be an active site directed reagent. The rationale is that the phosphopyridoxal moiety has a high affinity for the active site while the fluorinated dinitrophenyl moiety is the chemically reactive labelling reagent. It does react with the apoenzyme of aspartate aminotransferase but it only labels Cys-45 which is on the surface of the protein (Riva et al., 1979).

A few reduced Schiff based of fluorinated amino acids have been synthesized (Relimpio et al., 1975). One of these, has been used as a Fluorine-19 nmr reporter molecule in the identification of the pK of
the amino group of Lys-258. The pK was estimated to be about $8.0 \pm 0.5$, depending on the anion concentration (Martinez-Carrion et al., 1976).

The reduced Schiff base of pyridoxal 5'-phosphate with iodophenylalanine has been used as a heavy atom marker in the identification of the active site in the X-ray diffraction studies of chicken heart mitochondrial aspartate aminotransferase (Mavrides and Christen, 1978).
METHODS

**Instruments**

UV-visible absorption spectra were measured on a Cary 1501 double beam spectrophotometer equipped with a Cary-Datex digital output system. The spectra were recorded as a series of absorbance values at regular intervals of wavelengths. The sample cell holder is surrounded by a water jacket and its temperature was maintained at within ± 1°C of the desired temperature by circulation from a Lauda K-21B water bath. When following the time course of a reaction at single wavelengths, the monochrometer was disengaged from the drive motor and the chart was set to advance at a desired constant rate.

Circular Dichroism spectra were taken on a Jasco model ORD/UV-5 spectropolarimeter. The sample cell holder was also surrounded by a water jacket and its temperature was maintained to within ± 1°C of the desired value by circulation from a Forma-tempa Refrigerated/Heated Bath and Circulator.

All pH values were determined using a semimicro combination electrode connected to a Radiometer PHM64 pH-meter. A two-value standardization was performed at 7.00 and 4.00 or 7.00 and 10.00 with Fischer standard Buffer Solutions. Proton concentrations \([H]\) were defined as \([H] = 10^{-\text{pH}}\).

**Computation**

Whenever full spectra were used in the calculation, the programs used were those developed in Dr. Metzler's laboratory. Certain experi-
ments done at single wavelengths were analyzed statistically according to a mathematical model using nonlinear regression procedure SAS NLIN program. All computer-assisted calculations were carried out at the Computation Center of Iowa State University.

Materials

Chemicals used were of the best grade available commercially. L-aspartic acid, DL-α-methylaspartic acid, cysteine sulfenic acid, O-phosphoserine, α-ketoglutaric acid, adipic acid, oxalacetic acid, the reduced form of β-nicotinamide adenine dinucleotide (NADH), pyridoxal hydrochloride and pyridoxal 5'-phosphate were purchased from Sigma Chemical Co., malonic acid, L-glutamic acid were from Fischer Scientific Co., succinic acid was from Baker Chemical Co., 2-amino-3-phosphonopropionic acid was from Calbiochem, glutaric acid was from Aldrich Chemical Co., L-alanine was from Nutritional Biochemical Corp.

Triethanolamine hydrochloride (TEA.HCl) was prepared from the free base and was recrystallized three times by Mr. Robert D. Scott and Dr. Hiroshi Ueno. Erythro-β-hydroxy-L-aspartatic acid was a gift of Dr. W. Terry Jenkins.

Porcine heart malic dehydrogenase (MDH) was purchased from Sigma Chemical Co. in 50% glycerol containing 0.05 M phosphate pH7.5. It was dialyzed into 0.02 M TEA.HCl pH7.5 before use.
Figure 2. Methylation of PLP by dimethylsulfate at pH 9

The reaction was performed as described under Methods. 25 μL of the reaction mixture was diluted into 25 mL 0.05 M phosphate buffer pH 6.9. Curve a) reaction mixture before addition of dimethylsulfate, curve b) after completion of methylation.
Synthesis of N-methylpyridoxal 5'-phosphate

Pyridoxal 5'-phosphate (1.0 g, 4.1 x 10^{-3} mol) in 70 mL of water was placed in a 100 mL three-neck flask equipped with a combination electrode, magnetic stirrer, gas inlet and outlet. The solution was stirred and nitrogen was bubbled into it while its pH was maintained at 9 ± 1 by addition of 6N NaOH. When all the solid pyridoxal 5'-phosphate was dissolved, 0.38 mL portions (4.1 x 10^{-3} mol) of dimethyl sulfate were added, each followed by adjustment of the pH. During the course of the reaction, the pH decreased as a result of the hydrolysis of dimethyl sulfate; hence, an excess of this reagent was required. The progress of the reaction was followed by absorption spectroscopy with appropriate dilution of the reaction mixture into 50 mM phosphate buffer, pH 6.9 (Figure 2). At this pH, the ratio of absorbances at 394 nm to 331 nm (25.4 x 10^3 cm^{-1} to 30.2 x 10^3 cm^{-1}, A_{25.4/30.2} = 2.0). On the other hand, the completion of methylation was indicated by a value for A_{25.4/30.2} of 2.7. At this point, the addition of both dimethyl sulfate and NaOH was stopped, and the reaction mixture was stirred until no further change of pH was observed. The solvent was removed under reduced pressure between 40°C and 45°C. The residual red oil was taken up in a minimum amount of water and applied to an ion-exchange column (2.2 cm x 20 cm, Dowex 50W (H^+), 100-200 mesh, previously thoroughly washed with water). Air-free water was used for elution; it was prepared simply by bubbling nitrogen into the reservoir. The eluent was collected in 12 mL fractions at a rate of 30 mL/hr and assayed in
50 mM phosphate buffer, pH 6.9 (Figure 3). Two major bands were seen with $A_{25.4/30.2}$ greater than 3.0, the second of which contained the product. Thus, fractions 90-110 were pooled and the solvent was evaporated under reduced pressure. The residual lime-colored oil, upon grinding with acetone, yielded 350 to 400 mg (30-33%) of a yellowish, finely powdered solid.

**Synthesis of O-methylpyridoxal 5'-phosphate (O-MePLP)**

The hemiacetal form of pyridoxal was converted to O-methylpyridoxamine 5'-phosphate by the procedure of Besedina et al. (1971) followed by oxidation to O-MePLP by $\text{MnO}_2$ according to Pocker & Fischer (1969). The procedures of both Besedina et al. (1971) and Pocker & Fischer (1969) were published in detail and I was able to reproduce their results.

**Determination of pK values for N-MePLP**

Titrations of N-MePLP were performed according to the procedures of Johnson and Metzler (1970). Thus a $6.01 \times 10^{-4} \text{M}$ stock solution of N-MePLP in water was prepared. A 25.0 mL test solution was then made up in a volumetric flask with 5.0 mL of the stock solution, the appropriate amounts of buffer salts and $\text{NaClO}_4$ to maintain the final ionic strength at 0.2. A blank solution was also made up containing all components as the test solution except N-MePLP. Full spectra of the test solution were recorded against a blank solution in the reference beam in a Cary 1501 spectrophotometer equipped with a Cary-Datex digital output system.
Figure 3. Elution profile of ion-exchange chromatography in the purification of N-MePLP
Concurrently, the pH of the test solution was taken with a Radiometer model PHM 64 pH-meter.

The pK values of N-MePLP were evaluated by a computer-assisted data-fitting procedure according to Nagano and Metzler (1967). Spectra of N-MePLP at 13 pH values ranging from 3.8 to 10.00 were used as the input data. The output contains the pK values and spectra of the ionic forms of N-MePLP.

Analysis of absorption spectra using lognormal functions

It has been established (Siano & Metzler, 1969) that an electronic transition in the spectrum of a single chemical species can be fitted by a lognormal function: $\varepsilon(\nu) = \varepsilon_o \exp \left\{ \ln 2/\ln 10 \mu \nu \right\}^2 [\ln ((\nu-\nu_o)/(\rho^2-1)/W^o + 1)]^2$.

where parameters position $\nu_o$, height $\varepsilon_o$, width $W$, and skewness $\rho$ are defined in Figure 4. The difference between the molar absorptivities and the calculated ordinated values of the corresponding points of the sum of the lognormal functions are plotted against wave numbers to give the "difference plot." The lognormal curves and difference plots are generated by computer-assisted data fitting as described in the literature (Metzler et al., 1973).
Figure 4. Spectrum of the cation of 5-deoxypyradoxine with definitions of band parameters

The band position is $\nu$; the height is $\varepsilon$; $W$ is the width at the height $\varepsilon$/ and $\rho$ is the skewness equals to the ratio $b/a$. The points are experimental and the solid line is a lognormal curve fitted to the points. A "difference plot" shown at the top of the figure is a plot of the differences between the experimental points and the corresponding values on the fitted curve as a percentage of the peak height (Metzler et al., 1973. Reproduced with permission of the authors.)
Determination of equilibrium constants for the valyl Schiff base of N-MePLP

The procedures of Metzler et al. (1980) were followed. An aqueous stock solution of N-MePLP was made up at $5 \times 10^{-4}$ M. Then a test solution was made by pipetting 5.00 mL from the stock solution into a 25 mL volumetric flask containing the appropriate amounts of valine, buffer and NaClO$_4$ to maintain the final ionic strength at 0.2. A blank was prepared likewise except that N-MePLP was omitted. The complete spectrum of the test solution was recorded against the blank solution on a Cary 1501 spectrophotometer equipped with a Cary-Datex digital output system. Concurrently, the pH of the test solution was taken with a Radiometer model PHM64 pH meter. The equilibrium constants and spectra of the ionic forms were calculated by the computer-assisted data-fitting procedure of Metzler et al. (1980). The input data consisted of complete spectra of twelve solutions ranging in pH from 4.06 to 11.00 and in valine concentration from 0.016 M to 0.08 M together with pK values and spectra of ionic forms of N-MePLP obtained previously.

Enzyme

The $\alpha$-subform of cytosolic aspartate aminotransferase (AAT) from porcine heart muscle was prepared according to the methods of Martinez-Carrion et al. (1967). The product had a specific activity of $300 \pm 20$ $\mu$mol/min/mg at 25°C assayed according to the methods of Jenkins et al. (1959). The spectrum of the enzyme in 0.15 M acetate buffer pH 4.8 showed absorbance ratios of $A_{430}$ nm/$A_{430}$ nm = $3.9 \pm 0.1$ and $A_{430}$ nm/$A_{280}$ nm = $9.1 \pm 0.3$. 
The apo form of aspartate aminotransferase was prepared according to Scardi et al. (1963). This method involves conversion of the aldimine form of AAT to its corresponding amine form with an excess of glutamate at 0.2 M. The coenzyme was subsequently displaced from the protein by 0.5 M phosphate at pH 4.9. The product possessed 0.5% or less residual activity. Treatment with NaCNBH₃ at pH 8 in the presence of 10 mM α-ketoglutarate could further reduce the residual activity below 0.1% at the expense of some denaturation of the apo protein. The apo enzyme was harvested by ammonium sulfate precipitation. Before reconstitution, the protein was exhaustively dialyzed at pH 8, 4°C until the barium test for sulfate was negative.

**Aspartate Aminotransferase reconstituted with coenzyme analogs**

The apoenzyme of aspartate aminotransferase (usually at 10⁻⁴ M) was simply incubated with 1.2 equivalents of the desired coenzyme analog at pH 8, 25°C for 1 hour. The apoenzyme prepared as described above contains one mole/subunit of tightly bound phosphate (Verge et al., 1979). This phosphate is displaced upon binding of a coenzyme analog. Thus, before use, the reconstituted enzyme was dialyzed against three changes 250 mL of buffer at pH 8 to remove the phosphate ion.

**Measurement of enzymatic activity**

**Method 1:**

The procedures followed were essentially that of Jenkins et al., (1959). The enzyme (30 – 100 µL) was added with an adder mixer to a
thermostatted cuvet containing 3.0 mL of the appropriate assay mixture at
the desired temperature. The production of oxalacetate was observed at
280 nm (35.8 x 10^{-3} cm^{-1}) on a Cary 1501 spectrophotometer operating in the
synchronous mode. The molar absorptivity of oxalacetate was taken as
5.7 x 10^{-2} M^{-1} cm^{-1} (Velick & Vavra, 1962).

Method 2:
The procedure followed was essentially that for the coupled assay
of Karmen (1955). The production of exalacetate was measured by coupling
to its reduction by malic dehydrogenase (MDH):

\[
\text{aspartate} \xrightarrow{\text{AAT}} \text{oxalacetate} \xrightarrow{\text{MDH}} \text{malate}
\]
\[\text{NADH} \rightarrow \text{NAD}\]

One mole of NADH is converted to NAD for every mole of oxalacetate
reduced to malate.

The enzyme (AAT 20 - 60 μL) was added to a thermostated cuvet at
25° or 30°C containing 2.0 mL of an assay mixture containing various
concentrations of aspartate, α-ketoglutarate, buffer, 1 x 10^{-4} M NADH
and 8 units of malic dehydrogenase. An apparent activity was measured
in terms of the consumption of NADH as followed at 340 nm (29.4 x 10^{-3} cm^{-1}).
The molar extinction coefficient of NADH was taken as 6.03 x 10^{-3} M^{-1} cm^{-1}.
The malic dehydrogenase used contained as a contaminant some aspartate
aminotransferase activity which was determined as follows. Malic
dehydrogenase (8 units) was added to 2.0 mL of an assay mixture identical
to that described above except that it contained no malic dehydrogenase. Thus the activity of AAT of interest is given by activity of AAT = (apparent activity) - (contaminant activity). (2)

Theory of the interaction of anions with E.N-MePLP

The methods used to study the reactions of anions with E.N-MePLP are adapted from those developed by Bonsib et al. (1975) and by Jenkins (1980). A similar procedure of Fonda and Johnson (1970) was not used because the computer programs were outdated.

The spectral changes observed when anions were added to the aldimine form of AAT or E.N-MePLP were analyzed according to Scheme 6.

Scheme 6

Here X denotes the anion, HE and E are the protonated species of the enzyme, HEX and EX are the corresponding species of the enzyme-anion complex. All constants are defined with respect to dissociation of the complexes. Thus, $K_1 = [H][E]/[HE]$, $k_2 = [HE][X]/[HEX]$, $K_3 = [EX][H]/[HEX]$, $K_4 = [E][X]/[EX]$ and $K_1 K_2 = K_3 K_4$. The protonated species have an
absorption band at 430 nm (23.3 x 10^3 cm^{-1}) and their solutions are yellow. The total enzyme concentrations $[E_t]$ are given by

$$[E_t] = [HE] + [E] + [HEX] + [EX].$$

Together with the definitions of the dissociation constants,

$$[HE] = \frac{[X]}{1 + \frac{K_1}{K_2}[H]} + \frac{K_3}{K_2} \frac{1 + \frac{K_3}{[H]}[H]}{[X]}$$

$$[HEX] = \frac{[E_t]}{K_2} (1 + \frac{K_1}{[X]}[H]) + \frac{K_3}{[H]}$$

Let $\varepsilon_{he}$ and $\varepsilon_{hex}$ denote the molar absorptivities of HE and HEX, the absorbance at 430 nm is given by

$$A_{430} = \varepsilon_{he} [HE] + \varepsilon_{hex} [HEX] + r$$

$$= \frac{(\varepsilon_{he} K_2 + \varepsilon_{hex} [X]) [E_t] [H]}{K_2 [H] + K_2 K_1 + [H] [X] + K_3 [X] + r}$$

The term $r$ is the absorbance of the apo enzyme together with any small amounts of colored contaminant such as heme proteins not completely eliminated in the purification of the holo enzyme.

**Effects of acetate and chloride on proton dissociation of E.N-MePLP**

When titration with acid is performed at a constant concentration of anion, equation (3) simplifies to:
When $K_o$, the anion dependent apparent dissociation constant for the bound coenzyme is given as

$$K_o = \frac{K_2 K_1 + K_3 [X]}{K_2 + [X]}$$

(4)

(5)

$(A_x + r)$ is the maximum absorbance attainable at 430 nm for a given $[X]$. The values of $K_o$ were estimated according to equation (4) at several concentrations of $X$. Using these data $K_1$ to $K_3$ were then estimated from equation (5). Both calculations were made with the NLIN program.

The enzyme E.N-MePLP was concentrated to about 20 mg/mL and was dialyzed against two 500 mL changes of water at 4°C. The dialyzed enzyme was diluted to give an absorbance of 0.4 at 376 nm. No cloudiness resulted after dialysis against water. An 0.90 mL portion of the dialyzed enzyme diluted in water was mixed with 0.10 mL of an appropriate stock sodium acetate solution or a solution of KCl containing a known concentration of the sodium acetate. The pH of the mixture was brought down gradually by addition of 5 μL aliquots of acetic acid of known concentrations from a Hamilton Microliter Syringe. After thorough mixing with an addermixer, the spectrum was recorded at 30°C. A solution containing everything in the sample solution except the enzyme was used as blank as well as in the reference beam.
The proton concentration of the test solution was calculated by the equation $\text{pH} = \text{pK}^* + \log \frac{[\text{acetate}]}{[\text{acetic acid}]}$, where $\text{pK}^*$ is the pH given by a pH meter for a solution containing equal concentrations of acetic acid and acetate ion, the latter being identical in concentration to that in the test solution. These $\text{pK}^*$ values are collected in Tables 3 and 4. The pH of the test solution was also measured with a pH meter after all additions of acetic acid were completed, and was compared with the calculated value. It was found that the two values never differed by more than 0.04 pH units. Therefore, the error limits in this experiment were taken to be $\pm 0.05$ pH units.

Table 3. Effects of buffer concentration on the apparent pK ($\text{pK}^*$) of acetic acid

<table>
<thead>
<tr>
<th>Acetate ion (mM)</th>
<th>$\text{pK}^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>4.71</td>
</tr>
<tr>
<td>60</td>
<td>4.68</td>
</tr>
<tr>
<td>100</td>
<td>4.65</td>
</tr>
<tr>
<td>140</td>
<td>4.64</td>
</tr>
<tr>
<td>200</td>
<td>4.64</td>
</tr>
</tbody>
</table>
Table 4. Effects of KCl on pK* of acetic acid

<table>
<thead>
<tr>
<th>KCl (mM)</th>
<th>pK*</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>4.68</td>
</tr>
<tr>
<td>40</td>
<td>4.66</td>
</tr>
<tr>
<td>80</td>
<td>4.62</td>
</tr>
<tr>
<td>120</td>
<td>4.61</td>
</tr>
<tr>
<td>200</td>
<td>4.60</td>
</tr>
</tbody>
</table>

Each determination was made with an acetate ion concentration of 20 mM.

Binding of a dicarboxylate to E.N-MePLP

The apparent dissociation constant \( K_o \) was evaluated only at pH 7.2. At this pH, in 0.02 M chloride, an insignificant amount of HE is present. Using the relationship \( K_1 K_2 = K_3 K_4 \), equation (3) simplifies to:

\[
A_{430} = \frac{c_{hex} [E_t] [X]}{K_3 K_4 + [H] [X] + K_3 [X]} + r
\]

And at constant pH:

\[
A_{430} = \frac{A_H [X]}{K_o + [X]} + r
\]

The pH sensitive apparent dissociation constant \( K_o \) for dicarboxylate is given as:

\[
K_o = \frac{K_3 K_4}{K_3 + [H]}
\]
A solution of E.N-MePLP in 0.02 M TEA.HCl was adjusted to pH 7.20, 30°C. Portions of a 1 M solution of dicarboxylate at pH 7.0 were thoroughly mixed in. The spectrum was recorded after every addition using a 0.02 M TEA.HCl buffer solution as a blank in the reference cell. The pH of the sample immediately after titration was determined to be 7.23. The absorbance values at 430 nm (23.3 x 10³ cm⁻¹) were used to estimate $K_o$ according to equation (7) by the NLIN program.

The reaction of E.N-MePLP and glutamate pH 8.3

When E.N-MePLP is combined with a mixture of N-MePMP, glutamate and α-ketoglutarate, the reactions involved are summarized in the following scheme 7.

Scheme 7

Here, $E_L$ and $E_M$ represent the aldimine and amine forms of free E.N-MePLP, $E_L.kg$ and $E_M.glu$ are their nonproductive complexes with α-ketoglutarate (kg) and glutamate (Glu). The symbols, $E$ and $E.SO_4$ stand for the apoenzyme of AAT and its complex with $SO_4.X$ denotes all species of the mixture of enzyme substrate intermediary complexes in equilibrium. All reactions are reversible while the direction of the arrows indicate the definitions of the equilibrium
constants $K_4$ to $K_9$. Explicitly, $K_4 = [E_L][kg][E_L.kg]$; $K_5 = [E_L][Glu]/[X]$; $K_6 = [E_M][kg]/[X]$; $K_7 = [E_M][Glu]/[E_M.Glu]$; $K_8 = [E][N-\text{MePMP}]/[E_M]$ and $K_9 = [E][\text{SO}_4]/[E.SO_4]$. Let the molar absorptivities of $E_L.kg$, $E_L$, $X$, $E_M$, $E_M$.$Glu$ and $E$ be $\epsilon_k$, $\epsilon_1$, $\epsilon_x$, $\epsilon_m$, $\epsilon_g$ and $\epsilon_{\text{apo}}$.

Jenkins Method (Jenkins and D'Ari, 1966b) If no dissociation of N-MePMP occur, reactions 8 and 9 in Scheme 7 do not take place. Furthermore, if the reaction is performed by adding only glutamate to $E_L$, the amount of $\alpha$-ketoglutarate generated is low enough such that reaction is insignificant. Under these conditions, Scheme 7 simplifies to the following:

Scheme 8

The concentration of $\alpha$-ketoglutarate:

$$[kg] = [E_M] + [E_M.Glu]$$

$$= K_6[X]/[Glu](1+Glu/K_7).$$

Then $[E_M]+[E_M.Glu] = \sqrt{K_6[X](1+Glu/K_7)}$.

The total concentration of enzyme,

$$[E_L] = [E_L] + [X] + [E_M] + [E_M.Glu]$$

$$= (1+K_5/[Glu]) [X] + \sqrt{K_6[X](1+Glu/K_7)}$$
Since only E_L and X absorb at 376 nm (26.6 x 10^3 cm^-1), the absorbance at 376 nm is given as:

\[ A_{376} = \varepsilon_0[E_L] + \varepsilon_X[X] \]

\[ = [X] (\varepsilon_0 K_5/[Glu] + \varepsilon_X) \]

\[ [E_t] = A_{376} (1 + K_5/[Glu])/(\varepsilon_X + \varepsilon_0 K_5/[Glu]) + \sqrt{K_6(1 + [Glu]/K_7)}/A_{376}/(\varepsilon_X + \varepsilon_0 K_5/[Glu]) \]

now, dividing both sides by \( A_{376} \) and introducing the factor 1/c where

\[ 1/c = (1 + K_5/[Glu])/(\varepsilon_X + \varepsilon_0 K_5/[Glu]) \]  

We have an expression relating the inverse of the apparent molar absorptivity of the enzyme with glutamate and the dissociation constant,

\[ [E_t]/A_{376} = 1/c + \sqrt{K_6(1 + [Glu]/K_7)/A_{376}(1 + K_5/[Glu])} \]

In the reaction of E,N-MePLP and glutamate, E_M does dissociate to E and N-MePMP. However, examination of the time course of the reaction showed that reactions 5, 6, and 7 were completed in seconds while the release of the coenzyme took minutes (see Results section). As a first approximation, the values for \( A_{376} \) in equation (10) were obtained by extrapolating the absorbancies to zero time. To estimate \( K_5 \), in a primary plot the values of \( E_t/A_{376} \) were plotted against \( 1/A_{376} \) according to equation (10). Then, the reciprocals of the changes in the ordinate intercepts were plotted against [Glu] according to equation (9). The
negative abscissa of this secondary plot was numerically equal to \( K_5 \). To estimate \( K_6 \), the slopes of the primary plot were squared, multiplied by the respective ordinate intercepts and plotted against [Glu]. The negative abscissa gave the value of \( K_6 \).

**Saturation with N-MePMP in the presence of both glutamate and \( \alpha \)-ketoglutarate** If \( K_4 \) and \( K_7 \) are greater than the concentrations of \( \alpha \)-ketoglutarate and glutamate used, in the presence of saturating concentration of N-MePMP. Scheme 9 simplifies to the following:

\[
\text{Scheme 9}
\]

\[
\begin{array}{c}
\text{Glu} \\
E_L \xleftarrow{\epsilon} X \xrightarrow{\epsilon} E_M
\end{array}
\]

\[
K_5 \quad K_6
\]

According to this scheme, the total enzyme concentrations are given as:

\[
[E] = [E_L] + [X] + [E_M]
\]

\[
= [E_L](1+[Glu]/K_5 = K_6 [Glu]/K_5 [kg])
\]

\[
= [X](1 + K_5/ [Glu] + K_6/[kg])
\]

The absorbance at 377 nm\(^1\) is contributed only by \( E \) and \( X \). Therefore,

\[
A_{377} = \epsilon_1 [E_L] + \epsilon_x [X]
\]

\[
= [kg][E_L](\epsilon_1 K_5 + \epsilon_x [Glu])
\]

\[
(K_5 + [Glu])(kg + K_6 [kg])
\]

\[(11)\]

\(^1\)Here 377 nm is used, see Results.
Since α-ketoglutarate is generated in the reaction of glutamate with E.N-MePLP, an iterative procedure is employed to correct the total concentration of α-ketoglutarate. Using $K_5$ estimated, $[E_L]$ and $[X]$ can be calculated as follows:

\[
[E_L] = A_{377}/(\varepsilon_1 + \varepsilon_X[Glu]/K_5) \quad (12)
\]

\[
[X] = [E_L][Glu]/K_5 \quad (13)
\]

Let $[k_g']$ and $[k_g'']$ be the concentrations of α-ketoglutarate added and that generated, respectively, then

\[
[k_g''] = [E_M]
\]

\[
= [E_L] - ([E_L] + [X]) \quad (14)
\]

$K_5$ and $K_6$ can be evaluated again by equation (11) where the total concentration of α-ketoglutarate is given as

\[
[k_g] = [k_g'] + [k_g'']
\]

**Estimation of $K_9$, the dissociation constant for N-MePMP**

At concentrations of glutamate below 0.1 M, the amount of $X$ present is insignificant. Furthermore, if the concentration of α-ketoglutarate is one order of magnitude below $K_5$, Scheme 7 simplifies to the following:

...
At equilibrium, $K_e = \frac{[E_M][kg]}{[E_L][Glu]} = \frac{K_6}{K_5}$, and the concentrations of enzyme and α-ketoglutarate are given as

$$[E_t] = [E_L] + [E_M] + [E]$$
$$[kg] = [E_t] - [E_L]$$

Case 1: When only glutamate is added, the concentrations of $E_M$ and $E$ can be expressed as follows:

$$[E_M] = K_e [E_L] [Glu] / ([E_t] - [E_L])$$
$$[E] = [N-MePMP] = \sqrt{K_8 [E_M]}$$

Then the total enzyme concentrations become

$$[E_t] = [E_L] + \frac{K_e [E_L] [Glu]}{[E_t] - [E_L]} + \sqrt{K_8 K_e [E_L] [Glu] / ([E_t] - [E_L])}$$
An iterative procedure is needed to correct the amount of N-MePMP released from the enzyme. This is done by using the estimated value of $K_8$ to calculate the concentration of the apoprotein $[E']$.

$$[E'] = \frac{K_8 K_e [E_L][Glu]}{[E_L] - [E_L]}$$

(17)

The improved value of $K_8$ is then calculated as below

$$K_8 = \frac{([N-MePMP] + [E_L])[E']}{[E_F]}$$

(18)

The procedure is repeated until a constant value of $K_8$ is found.
Case 3: A low concentration of glutamate is equilibrated with E.N-MePLP and α-ketoglutarate.

The concentrations of E and \( E_M \) are given as:

\[
[E] = [N-MePMP] \\
[E_M] = K_e [E_L][Glu]/[kg] \\
K_8 = \frac{[E][E_L][Glu]}{[kg]} \times \frac{[E]}{[E_L][Glu]} \\
\]

(19)

Estimation of \( K_{10} \), the dissociation constant for sulfate  

The reaction is carried out at low concentrations of glutamate. Under this condition, the concentration of X is insignificant, and reactions 4 and 7 can also be neglected. Scheme 7 becomes the following:

\[
\begin{align*}
\text{Scheme 11} \\
\text{Glu} & \quad \text{kg} & \quad \text{N-MePMP} & \quad \text{SO}_4 \\
E_L & \xrightarrow{K_e} E_M & \xrightarrow{K_8} E & \xrightarrow{K_9} E\text{SO}_4 \\
\end{align*}
\]

At equilibrium, the following hold:

\[
[E_L] = [E_M] + [E] + [E\text{SO}_4] \\
[kg] = [E_L] - [E_L] \\
[N-MePMP] = [E] + [E\text{SO}_4] = [E](1 + [SO_4]/K_9)
\]
From the definition of $K_8$ and $K_e$

$$[E][N\text{-MePMP}] = K_8[E_M]$$

$$= K_8 K_e [E_L][Glu]$$

$$= [E]^2 \left(1 + \frac{[SO_4]}{K_9}\right)$$

Together with the above expression for $[E_\ell]$

$$K_8(1 + [SO_4]/K_9) = \frac{\left([E_\ell] - [E_L]\right)^2 - K_e [E_L][Glu])^2}{\left([E_\ell] - [E_L]\right)(K_e [E_L][Glu])} \quad (20)$$

If the concentration of sulfate used is larger than $K_9$, an iterative procedure is used to refine the value of $K_9$. Using the estimated value of $K_9$, the concentration of the apoenzyme is given as below:

$$[E] = \frac{K_8}{[E] + [E\cdot SO_4]} \frac{K_e [E_L][Glu]}{[E_\ell] - [E_L]}$$
Applying the quadratic formula:

\[
[E] = \frac{1}{2} \sqrt{1 + 4K_8 \left( \frac{K_{eL}[\text{Glu}]}{[E_L] - [E_L]} \right)^2 - 1}
\] (21)

If \([E]\) is found to be insignificantly small, this means that the following ion exchange reaction is involved:

Scheme 12

\[
\begin{array}{c}
\text{Glu} \\
\text{N-MePMP}
\end{array}
\xrightarrow{K_x}
\begin{array}{c}
\text{E}_L \\
\text{SO}_4^-
\end{array}
\rightarrow
\begin{array}{c}
\text{E}.\text{SO}_4
\end{array}
\]

Where \(K_x = \frac{[\text{E}.\text{SO}_4][\text{N-MePMP}][k_g]}{[E_L][\text{Glu}][\text{SO}_4^-]}\) (22)

Let \([\text{SO}_4^-']\) be the concentration of sulfate added, the concentration of sulfate present at equilibrium \([\text{SO}_4^-]\) is given as

\([\text{SO}_4^-] = [\text{SO}_4^-'] - [E_L] + [E_L].\) Thus, \(K_x = ([E_L] - [E_L])^3/[E_L][\text{Glu}][\text{SO}_4^-]\)

\[= \frac{K_{e8}K_{59}}{K_{x9}} = \frac{K_{e8}}{K_9}\]

\[K_9 = \frac{K_{e8}}{K_x}\] (23)
Determination of $k_o$, the pseudo first order rate constant of a reaction

The time course of the reaction was followed spectrophotometrically. The half life ($t_{1/2}$) of the reaction was estimated from a plot of $\log_{10} |A_t - A_\infty|$ against time, where $A_t$ and $A_\infty$ are absorbance value at time $t$ and time = $\infty$. The value for $k_o$ were calculated from the equation

$$k_o = \frac{\ln 2}{t_{1/2}}$$

Equation for the spectrophotometric titration of quinonoid intermediate at 480 nm ($20.8 \times 10^3 \text{ cm}^{-1}$)

The reaction is simply $\text{HE} \rightarrow \text{E} + \text{H}$; however in this case HE is colorless and only E has absorption at 480 nm.

$$K_o = [\text{H}][\text{E}]/[\text{HE}] \quad [E_t] = [\text{HE}] + [\text{E}]$$

$$= (1 + [\text{H}]/K_o)[\text{E}]$$

Let $\varepsilon$ be the molar absorptivity of E, then the absorbance at 480 nm is given as

$$\Delta A_{480} = \varepsilon[E]$$

$$= A_m/(1 + [\text{H}]/K_o)$$

(25)

where $A_m = \varepsilon[E_t]$. 
This can be linearized in the form of a scatchard plot: 
\[ \Delta A_{480}^0[H] = -\Delta A_{480}^0 K_o + A K_m^0 \]  

(26)

**Derivation of** \( k_o \), **the pseudo first order rate constant for the decay of two species at equilibrium to a common product**

For the reaction depicted in Scheme 13,

**Scheme 13**

Let the apparent proton dissociation constant for \( HE \rightarrow H + E \) be 
\[ K_o = \frac{[H][Q]}{[HQ]} \]

Then, 
\[ [Q] + [HQ] = E(1 + [H]/K_o) \]

\[
\frac{d[P]}{dt} = -\left( \frac{d[Q]}{dt} + \frac{d[HQ]}{dt} \right)
\]

\[ = -(1 + [H]/K_o) \frac{d[Q]}{dt} \]

Also 
\[
\frac{d[P]}{dt} = k_B [Q] + k_A [HQ]
\]
\[ \frac{d[E]}{dt} = \frac{\left( k_B K_o + k_A [H] \right)[Q]}{K_o + [H]} \]

\[ [Q] = [Q_0] e^{-k_o t} \]

where \( k_o = \frac{k_B K_o + k_A [H]}{K_o + [H]} \)

Crystallization of E.N-MePLP and E.O-MePLP

The method of McPherson (1976) was used. Seeding was required to grow large platelike crystals. E.N-MePLP or E.O-MePLP was dialyzed against 0.08 M acetate, pH 5.4, and concentrated to 70 mg/mL. It was mixed with an equal volume of the same buffer containing 8% polyethylene glycol, MW 6000. Portions of 1 to 2 mg of protein were delivered into wells of a glass spot plate. The spot plate was supported on a petri dish containing 25 mL of 0.08 M acetate buffer pH 5.4 containing 8% polyethylene glycol, MW 6000. The glass plate and petri dish combination were sealed in a plastic sandwich box. Seeding was done with a small crystal of native AAT. E.N-MePLP or E.O-MePLP will grow on the seed, but usually some nucleation sites also developed independently. The latter yielded small platelike crystals which were used as seeds for growing larger crystals.

Crystal Spectra of E.N-MePLP

The procedures of Metzler et al. (1979) are used. Since the crystals
of E.N-MePLP have not been examined by X-ray diffraction studies, the crystallographic axes are presumed to be oriented identically as those in the native enzyme. A platelike crystal of E.N-MePLP is placed in the sample compartment of the spectrophotometer such that the plane-polarized light travels along the \( b \)-axes of the crystals. Two spectra for each crystal are recorded, one with the plane of the polarized light parallel to the \( a \)- and the other to the \( c \)-axis.
RESULTS

Spectra and pKa values of N-Methylpyridoxal phosphate

The equilibria involved in the stepwise proton dissociation from N-MePLP together with those of PLP are shown in Scheme 14. However, it must be remembered that each aldehyde form is in equilibrium with its hydrate which is not shown in Scheme 14. Thus, the pK values given above the arrows in Scheme 14 are the negative logarithms of the overall stepwise proton dissociation constants K (Harris et al., 1976).

For the purpose of discussing equilibria of different ionic forms of both the phosphorylated and dephosphorylated analogs of vitamin B6, a new set of symbols is used to designate the ionic forms (Scheme 15). The small letter "a" is used to designate the aldehyde form and "h" the corresponding hydrate of a. The phosphorylated forms of a and h are designated as ap and hp. The charge possessed by each ionic form is shown by superscripts above the letter. Figure 5 shows several spectra of N-MePLP in the acidic pH range. These together with twelve other spectra at higher pH values served as input data for the simultaneous computation of the pK's and spectra of individual ionic species of N-MePLP. At pH greater than 6, there is some increase for the band at 333 nm (30.0 x 10^3 cm^-1) and a concomitant decrease for that at 25.4 kK. Otherwise, increasing the pH up to 12 has little effect on the spectrum of N-MePLP. Figure 6 shows spectrophotometric titration at three different wave numbers, 394 nm (25.4 x 10^3 cm^-1), 333 nm (30.3 x 10^3 cm^-1) and 298 nm (33.6 x 10^3 cm^-1). These data are consistent with there being two ionization steps for N-MePLP corresponding to pK values of 3.80 and
Scheme 14

H2P  \[ \rightleftharpoons \]  HP  \[ \rightarrow \rightarrow \]  P

\[ pK_1 = 3.68 \]

H3P  \[ \rightleftharpoons \]  H2P  \[ \rightleftharpoons \]  HP  \[ \rightarrow \rightarrow \]  P

\[ pK_1 = 3.62 \]
\[ pK_2 = 6.10 \]
\[ pK_3 = 8.33 \]
Scheme 15

\[ \text{CHO} \quad \text{CHO} \quad \text{CHO} \]

\[ \text{HI} \quad 1\text{PO}_3\text{H} \quad \text{OPO}^\text{H} \]

\[ \text{K}_{1a} \quad \text{K}_{2a} \]

\[ \text{CHO} \quad \text{CHO} \quad \text{CHO} \]

\[ \text{a}^+ \text{p}^- \quad \text{a}^+ \text{p}^- \quad \text{a}^+ \text{p}^- \]

\[ \text{R}_{1h} \quad \text{R}_{2h} \quad \text{R}_{3h} \]

\[ \text{HO} \quad \text{OH} \quad \text{HO} \quad \text{OH} \quad \text{HO} \quad \text{OH} \]

\[ \text{h}^+ \text{p}^- \quad \text{h}^+ \text{p}^- \quad \text{h}^+ \text{p}^- \]
Scheme 16

\[
\text{H}2\text{P} \quad pK = 3.68 \\
\text{H}2\text{P}^+ \\
\text{H}2\text{P}^{2+}
\]

\[
\text{HP} \quad pK = 6.00 \\
\text{HP}^+ \\
\text{HP}^{2+}
\]

\[
\frac{\text{(CH}_3\text{)}_2\text{CHCH(NH}_3^+\text{)}\text{COOH}}{\text{H}2\text{L}} \quad pK = 2.32 \\
\frac{\text{(CH}_3\text{)}_2\text{CHCH(NH}_3^+\text{)}\text{COO}^-}{\text{HL}} \quad pK = 9.62 \\
\frac{\text{(CH}_3\text{)}_2\text{CHCH(NH}_3^+\text{)}\text{COO}^{2-}}{\text{L}}
\]

\[
\log K_f = 1.58
\]

\[
\text{R} = (\text{CH}_3\text{)}_2\text{CHCHCOO}^-
\]
6.00. They are assignable to the 3-phenolic and 5'-phosphate. The spectra for the three ionic species of N-MePLP are shown in Figure 7. These have been analyzed by resolution with lognormal functions as described in METHODS. The "difference plot" generated is shown in Figure 8. The spectral band parameters for the ionic forms of N-MePLP together with those of several vitamin B₆ analogs are collected in Table 5.

Tautomerization and hydration ratios and microscopic dissociation constants

Scheme 15 shows the equilibria involved for the hydrates of each ionic species of N-MePLP. $K_{1a}$ and $K_{2a}$ are microscopic dissociation constants and $R_{1h}$, $R_{2h}$ and $R_{3h}$ are tautomeric ratios defined as:

$$R_{1h} = \frac{h^+P^-}{a^+P^-}; R_{2h} = \frac{h^+P^-}{a^+P^-}; R_{3h} = \frac{h^+P^=}{a^+P^=}.$$  

These are related to the stepwise dissociation constants of Scheme 15 as follows:

$$K_1 = K_{1a} \frac{(1 + R_{2h})}{(1 + R_{1h})},$$
$$K_2 = K_{2a} \frac{(1 + R_{3h})}{(1 + R_{2h})}. \quad (2)$$

In the absence of a detailed temperature study of N-MePLP, the tautomeric ratios were estimated using the molar areas of pyridoxal 5'-phosphate and of N-methylpyridoxal. These values together with the microscopic dissociation constants of N-MePLP are collected in Table 6.

Schiff Base of N-MePLP

When the amino acid valine is added to N-MePLP, a Schiff base is formed. The equilibria involved are shown in Scheme 16. The underlined values given are found by the computer-assisted data fitting procedure described under METHODS. In Scheme 16, $pK$ values are again the negative logarithms of proton dissociation constants. $K_F$ is a pH independent
Figure 5. Examples of spectra of N-MePLP at different pH

The points are experimental and the solid lines are calculated spectra using an observed pK of 3.68. The pH of the solutions are 5.23 (*), 3.96 (o), 3.67 (+), 2.95 (o) and 1.43 (X).
Figure 6. Fixed wavenumber spectrophotometric titration curves for N-MePLP

The solid lines are theoretical and the points are experimental

394 nm (25.4 x 10^3 cm\(^{-1}\), x),
333 nm (30.0 x 10^3 cm\(^{-1}\), o) and
293 nm (33.6 x 10^3 cm\(^{-1}\), +)
Figure 7. Spectra for the ionic forms of N-MeFLP as resolved using lognormal functions

The lines are the spectra defined by lognormal functions while the points are molar absorptivities determined for each ionic form using the spectra of N-MeFLP at 17 pH values. A) H₂P, B) HP and C) P forms.
Figure 7 (Continued)
Figure 8. Difference plot for the ionic species of N-MePLP

A) H2P form,
B) HP form and
C) P form
Figure 8 (Continued)
Figure 8 (Continued)
Table 5. Band shape parameters for PLP and N-MePLP

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ionic form</th>
<th>$v_o \times 10^{-3}$ cm$^{-1}$</th>
<th>$\epsilon_o \times 10^{-3}$</th>
<th>$W(X \times 10^{-3}$ cm$^{-1}$</th>
<th>Area</th>
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<td>4.60</td>
<td>1.24</td>
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<tr>
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<td>$h^+ -$</td>
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<td>6.17</td>
<td>3.24</td>
<td>1.55</td>
</tr>
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<td>6.55</td>
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<td>4.67</td>
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<td>1.46</td>
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</tr>
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<td>6.61</td>
<td>3.25</td>
<td>1.46</td>
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<td>1.36</td>
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</table>

\(^a\) This work.

\(^b\) Harris et al., (1976).
formation constant defined as follows:

\[ K_f = \frac{[HPL]}{[HL][P]} \]  

(30)

Thus, for the valyl Schiff base of N-MePLP, the value of pK for proton dissociation from 4'-imine group was found to be 9.60 while the value of \( \log K_f \) is 1.58. The input data are the spectra of the ionic forms of N-MePLP and for valine together with 12 spectra of different mixtures of valine and N-MePLP, several of which are shown in Figure 9. Figure 10 is a plot of the pH-dependent apparent formation constant \( K_{pH} \) against pH where \( K_{pH} \) is defined as follows:

\[ K_{pH} = \frac{[\text{total Schiff Base}]}{[\text{total N-MePLP}][\text{total valine}]} \]  

(31)

Figure 11 presents the spectra for the ionic species of valyl Schiff base of N-MePLP. These spectra have been analyzed by lognormal functions as described under METHODS. The "difference plots" for the ionic forms are shown in Figure 12. The spectral band parameters for the ionic forms of the Schiff base of N-MePLP are collected in Table 7.

Table 8 compares log \( K_f \) values for Schiff bases of various vitamin \( B_6 \) analogs. Table 9 compares pKa values for the dissociation of the imino proton of valyl Schiff bases of several vitamin \( B_6 \) analogs.

**Nonenzymatic Transamination**

PLP is able to undergo reversible transamination with glutamate (Metzler and Snell, 1952). The reaction is catalyzed by metal salts (Metzler et al., 1954). Likewise, the reversible transamination of glutamate to \( \alpha \)-ketoglutarate was tested with both N-MePLP and N-MePMP at pH 8.3 (Figure 13). At 30°C, the reaction was very slow and took three days to come to equilibrium even in the presence of aluminum ion.
Figure 9. Examples of spectra of N-MePLP in the presence of 0.08 M valine at different pH

The pH values of the solutions are 6.86 (+), 9.96 (x) and 11.00 (o). The points are experimental and the solid lines are calculated spectra using the formation constants and pK values given in Scheme 14 under Results.
Figure 10. Variation of $K_{pH}$ as a function of pH
Figure 11. Ionic forms of the Schiff base of N-MePLP with valine as resolved by lognormal functions

The solid lines are lognormal functions while the points are calculated molar absorptivities of ionic forms.

A) H2PL,
B) HPL and
C) PL
Figure 11 (Continued)
Figure 12. Difference plots for ionic forms of the valyl Schiff base of N-MeFLP

A) H2PL,
B) HPL and
C) PL
Figure 12 (Continued)
Figure 12 (Continued)
Figure 13. Time course for nonenzymatic transamination of N-MePLP

93 μM N-MePLP was incubated with 0.02 M glutamate and 0.2 M α-ketoglutarate in 0.02 M triethanolamine hydrochloride, pH 8.3, 30°C in the presence of 5 x 10^{-5} M KAlSO_4 under nitrogen (o). 37 μM N-MePMG was incubated with 0.02 M glutamate and 0.2 M α-ketoglutarate in 0.02 M triethanolamine hydrochloride, pH 8.3, 30°C in the presence of 5 x 10^{-5} M KAlSO_4 under nitrogen (Δ).
Table 6. Tautomeric and microscopic dissociation constants for PLP and N-MePLP

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration fractions of ionic species</th>
<th>Tautomeric constants</th>
<th>Micros. diss. constants</th>
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<tr>
<td></td>
<td>[h+ p-] [a+ p-] [h- p+] [a- p+] [h+ p+] [a+ p+]</td>
<td>R1h R2h</td>
<td>K1a K2a</td>
</tr>
<tr>
<td>N-MePLP</td>
<td>0.72 0.24 0.17 0.71 0.21 0.17 3.00 0.23(a+ p-) 12.03 5.73</td>
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<tr>
<td>PLP</td>
<td>0.76 0.24 0.15 0.54 0.16 0.53 3.20 0.28(a+ p-) 8.30 5.88 0.48(a- p+)</td>
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</table>

*a Calculated using molar areas of PLP (Harris et al., 1976).

b Values taken from Harris et al., (1976).
Table 7. Band shape parameters for the Schiff bases of PLP and of N-MePLP with valine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ionic form</th>
<th>Peak position $\nu_0$ (x $10^{-3}$ cm$^{-1}$)</th>
<th>Peak height $\epsilon_0$ (x $10^{-3}$)</th>
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<th>Skewness $\rho$</th>
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<tr>
<td>N-MePLP</td>
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$^a$Metzler et al. (1980).
Table 8. Log $K_f$ values for Schiff bases of vitamin $B_6$ derivatives and valine

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<th>$B_6$-derivative</th>
<th>log $K_f$</th>
<th>$B_6$-derivative</th>
<th>log $K_f$</th>
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<td><img src="image1" alt="Chemical Structure 1" /></td>
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</table>
$K_f$ is the Schiff base formation constant defined for the reaction involving the following ionic species.

Where $R_1 = -H, -CH_3$
$R_2 = -CH_2OH, -CH_3, -CH_2PO_3^-$
$R_3 = -CH(CH_3)_2$
Table 9. Values of $-\log K_a^a$ for the imino nitrogen fo Schiff bases of vitamin B$_6$ analogs with valine

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<th>Schiff base</th>
<th>$-\log K_a$</th>
<th>Schiff base</th>
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<td>10.49$^b$</td>
<td><img src="image2.png" alt="Schiff base 2" /></td>
<td>12.16$^b$</td>
</tr>
<tr>
<td><img src="image3.png" alt="Schiff base 3" /></td>
<td>9.50$^b$</td>
<td><img src="image4.png" alt="Schiff base 4" /></td>
<td>9.60</td>
</tr>
</tbody>
</table>
\[ \text{R is the proton dissociation constant for the following reaction.} \]

\[ \text{\( R = \text{O}^\text{C-CH-CHCCH}\)} \]

\[ \text{\( \text{\text{\textit{Metzler e}}^{12,13}} \) } \]

\[ \text{\( \text{\textit{Ka} is the proton dissociation constant for the following reaction.} \)} \]

\[ \text{\( \text{\textit{Ka}} \) \( \text{\( \text{\textit{Metzler et al. (1980).}} \}}} \]

\[ \text{\( \text{where } R = \text{O}_2\text{C-CH-CH(CH}_3\text{)}_2 \)}} \]

\[ \text{\( \text{\textit{Metzler et al. (1980).}} \)}} \]
The total amount of aluminum ion added was almost stoichiometric with N-MePLP (1 x 10^{-4} M). At pH 8.3, the majority of the aluminum ion was probably not free but complexed by glutamate. From the absorbance at 400 nm (25.0 x 10^3 cm^{-1}) at equilibrium the equilibria for the transaminate was calculated as

$$K_t = \frac{[\alpha\text{-ketoglutarate}][N\text{-MePMP}]}{[\text{glutamate}][N\text{-MePLP}]} = 41\pm 2$$

Aspartate aminotransferase reconstituted with N-MePLP (E.N-MePLP)

When one equivalent of N-MePLP was combined with about 60 µM of apo AAT in 0.02M TEA.HCl pH 8 at 25°C, within 15 min the absorption band of the free coenzyme analog at 400 nm (25.0 x 10^3 cm^{-1}) was completely shifted to a new band at 376 nm (26.6 x 10^3 cm^{-1}). As in native AAT, the protein-bound coenzyme is a colorimetric pH indicator. At low pH, a solution of E.N-MePLP is yellow with an absorption band centered at 430 nm (23.3 x 10^3 cm^{-1}). Figure 14 shows spectrophotometric titration of E.N-MePLP. The apparent pK (pKo) associated with color transition is 5.4. From these data, spectra for the ionic forms are calculated and shown in Figures 15 and 16. The band parameters for the spectra are collected in Table 10.

The circular dichroism spectra of E.N-MePLP were also checked at pH 8 and pH 5.4. The results of Furbish et al. (1969) were confirmed in that both absorption bands at 430 nm and 376 nm exhibit positive dichroism with the value of ΔA/A = 2.0± 0.1.

Effects of anions on proton dissociation of E.N-MePLP

The value of the apparent pK for E.N-MePLP (5.4) found above is substantially different from 6.3, the value usually cited for the
Figure 14. Spectrophotometric titration of E.N-MEPLP

A) Effects of pH on the spectrum of the enzyme. The enzyme was dissolved in 0.02 M triethanolamine hydrochloride, pH 8.3. The pH was decreased by addition of 2 M acetic acid. The pH values of the solutions are 4.3 (X), 5.0 (o), 5.4 (+), 6.0 (m) and 8.3 (*).

B) Titration curves at fixed wavelengths, 430 nm (23.3 x 10^{-3} cm^{-1}, x) and 376 nm (26.6 x 10^{-7} cm^{-1}, o). The points are experimental and the solid lines are calculated using an apparent pK (pK_a) of 5.41.
Figure 14 (Continued)
Figure 15. Ionic forms of E.N-MePLP

The points are calculated values of molar absorptivity from the data of Figure 14.

A) Protonated form and
B) Deprotonated form
Figure 15 (Continued)
Figure 16. Difference plots of the ionic forms of E.N-MePLP

A) Protonated form and
B) deprotonated form
Figure 16 (Continued)
Table 10. Band shape parameters of E.N-MePLP and native AAT

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Form</th>
<th>Peak position (x 10^{-3} cm^{-1})</th>
<th>Peak height (x 10^{-3})</th>
<th>Width (x 10^{-3} cm^{-1})</th>
<th>Skewness</th>
<th>Area a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native AAT^a</td>
<td>Protonated</td>
<td>23.2</td>
<td>5.80</td>
<td>3.80</td>
<td>1.54</td>
<td>243.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.5</td>
<td>1.26</td>
<td>4.00</td>
<td>1.30</td>
<td>54.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29.7</td>
<td>1.01</td>
<td>4.00</td>
<td>1.50</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>Deprotonated</td>
<td>27.5</td>
<td>7.40</td>
<td>3.93</td>
<td>1.45</td>
<td>317.7</td>
</tr>
<tr>
<td>EN-MePLP</td>
<td>Protonated</td>
<td>23.3</td>
<td>7.60</td>
<td>3.98</td>
<td>1.70</td>
<td>364.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.0</td>
<td>1.23</td>
<td>4.00</td>
<td>1.50</td>
<td>54.2</td>
</tr>
<tr>
<td></td>
<td>Deprotonated</td>
<td>26.7</td>
<td>8.91</td>
<td>3.72</td>
<td>1.42</td>
<td>361.5</td>
</tr>
</tbody>
</table>

^aMetzler, private communication, 1981.
apparent pK of native AAT (Jenkins and Sizer, 1959). The colorimetric pH indicator properties of the bound coenzyme are sensitive to anions. Therefore, it was of interest to ascertain whether the discrepancy in the values of pK\(^o\) is due to a change in the anion affinity of the enzyme as a result of N-methylation of the coenzyme. Acid titrations were performed on E.N-MePLP at several concentrations of acetate and chloride as described in METHODS. The values of pK\(^o\) obtained are summarized in Table 11. Sample data for spectrophotometric titration in acetate and chloride are shown in Figures 17 to 19 and Figures 20 to 22, respectively.

When the data were analyzed according to Equation (5) given in METHODS, the reactions of acetate with E.N-MePLP cannot be described by Scheme 6. Despite this, extrapolation of K\(^o\) to zero acetate concentration, gave a value whose pK is 5.13 ± 0.01 (Figure 23a). Comparatively, 0.02 M acetate apparently has only a small effect on this value. The acid titration of E.N-MePLP at varying concentrations of chloride was performed in the presence of 0.02M sodium acetate. The value of K\(^o\) thereby obtained is described by equation (5), Figure 23b yielding K\(_1\) to K\(_3\) summarized in Table 12.

Formation of the nonproductive complexes of E.N-MePLP with dicarboxylates

The addition of up to 0.05M α-ketoglutarate to E.N-MePLP in 0.02M TEA.HCl pH 8.3 did not result in any yellow color suggesting that the protonated enzyme-dicarboxylate complex is not present at that pH. At pH 7.2, addition of a few mM of α-ketoglutarate does cause a small amount of absorbance at 430 nm (23.3 x 10\(^3\) cm\(^{-1}\)). Thus,
Figure 17. Spectrophotometric titration of E.N-MePLP in the presence of mM acetate anion

The symbols '§' are experimental and '*' are calculated according to equation 4 under Methods using an observed pK of 5.14.
Figure 18. Spectrophotometric titration of E.N-MePLP in the presence of 60 mM acetate anion

The symbols '$' are experimental and '*' are calculated according to equation 4 under Methods using an observed pK of 5.19.
Figure 19. Spectrophotometric titration of E.N-MePLP in the presence of 200 mM acetate anion

The symbols '§' are experimental and '*' are calculated according to equation 4 under Methods using an observed pK of 5.56.
Figure 20. Spectrophotometric titration of E.N-MePLP in the presence of 20 mM acetate anion and 20 mM chloride ion

The symbols '$$' are experimental and '*#' are calculated according to equation 4 under Methods using an observed pK of 5.41.
Figure 21. Spectrophotometric titration of E.N-MePLP in the presence of 20 mM acetate anion and 80 mM chloride ion

The symbols '§' are experimental and '*' are calculated according to equation 4 under Methods using an observed pK of 5.64.
Figure 22. Spectrophotometric titration of E.N-MePLP in the presence of 20 mM acetate anion and 120 mM chloride ion

The symbols '§' are experimental and '*' are calculated according to equation 4 under Methods using an observed pK of 5.84.
Figure 23. Effects of anions on the apparent proton dissociation constant ($K_o$) of E.N-MePLP

A) Acetate and
B) chloride

The symbols '§' are experimental and '*' are calculated according to equation 5 under Methods using the data presented in Table 12.
Figure 23 (Continued)
Figure 24. Titration of E.N-MePLP with α-ketoglutarate at 30°C

Aliquots (1-10 µL) from a solution of α-ketoglutarate (0.5 M, pH 7) were added with a Hamilton microsyringe to a 1.0 mL solution of E.N-MePLP (94 µM) in 0.02 M triethanolamine hydrochloride, pH 7.23. After mixing with an addermixer, the absorbance was read at 430 nm (23.3 x 10^{-3} cm^{-1}). The symbols '*' are experimental and '$' are calculated according to equation 7 under Methods using an apparent dissociation constant, $K_0$, of 4.1 mM.
Figure 25. Titration of E.N-MePLP with glutarate at 30°C

Aliquots (1-10 μL) from a solution of glutarate (1 M, pH 7) were added with a Hamilton microsyringe to a 1.0 mL solution of E.N-MePLP (94 μM) in 0.02 M triethanolamine hydrochloride, pH 7.23. After mixing with an addermixer, the absorbance was read at 430 nm (23.3 x 10^3 cm⁻¹). The symbols '*' are experimental and '§' are calculated according to equation 7 under Methods using an apparent dissociation constant, Kₐ, of 7.8 mM.
$- \log_{10} \text{[GLUTARATE (M)]}$
Figure 26. Titration of E.N-MePLP with succinate at 30°C

Aliquots (1-10 μL) from a solution of succinate (1M, pH 7) were added with a Hamilton microsyringe to a 1.0 mL solution of E.N-MePLP (94 μM) in 0.02 M triethanolamine hydrochloride, pH 7.23. After mixing with an addermixer, the absorbance was read at 430 nm (23.3 x 10^3 cm^-1). The symbols '*' are experimental and '$' are calculated according to equation 7 under Methods using an apparent dissociation constant, \( K_o \), of 9.8 mM.
$\log_{10} \text{[Succinate (M)]}$
Figure 27. Titration of E,N-MePLP with adipate at 30°C

Aliquots (5-30 μL) from a solution of adipate (1 M, pH 7) were added with a Hamilton microsyringe to a 1.0 mL solution of E,N-MePLP (94 μM) in 0.02 M triethanolamine hydrochloride, pH 7.23. After mixing with an addermixer, the absorbance was read at 430 nm (23.3 × 10^3 cm⁻¹). The symbols '*' are experimental and 'S' are calculated according to equation 7 under Methods using an apparent dissociation constant, K, of 21 mM.
Table 11. Effects of anion on the observed $pK$ ($pK_o$) of E.N-MePLP and native AAT

<table>
<thead>
<tr>
<th>Anion</th>
<th>Conc'n of anion (M)$^a$</th>
<th>$pK_o$</th>
<th>E.N-MePLP</th>
<th>Native AAT$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>0.02</td>
<td>5.14 ± 0.05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>5.19 ± 0.05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>5.28 ± 0.05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.14</td>
<td>5.35 ± 0.05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>5.56 ± 0.05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Chloride$^b$</td>
<td>0.00</td>
<td>5.14 ± 0.05</td>
<td>5.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>5.41 ± 0.06</td>
<td>5.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>5.48 ± 0.05</td>
<td>6.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>5.64 ± 0.05</td>
<td>6.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>5.70 ± 0.05</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>5.84 ± 0.05</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

$^a$This is the concentrations of chloride and acetate anion present in the reaction mixture.

$^b$All titrations in the presence of chloride were carried out in 0.02 M acetate anion as described under METHODS.

Table 12. Effects of anions on the pK of E.N-MePLP and of native AAT and the dissociation constants for the anions

<table>
<thead>
<tr>
<th>Constants</th>
<th>E.N-MePLP</th>
<th>Native AAT(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloride</td>
<td>Chloride</td>
</tr>
<tr>
<td>pK(_1)</td>
<td>5.14 ± 0.05</td>
<td>5.73 ± 0.12</td>
</tr>
<tr>
<td>K(_2) (mM)</td>
<td>20.0 ± 4.0</td>
<td>20.0 ± 1.0</td>
</tr>
<tr>
<td>pK(_3)</td>
<td>5.98 ± 0.10</td>
<td>6.76 ± 0.04</td>
</tr>
<tr>
<td>K(_4) (mM)</td>
<td>138.0 ± 96.0</td>
<td>216.0 ± 29.0</td>
</tr>
</tbody>
</table>

\(^a\)Jenkins (1981).
### Table 13. Apparent dissociation constants $K_o$ for dicarboxylates in their complexes with enzymes

<table>
<thead>
<tr>
<th>Dicarboxylates</th>
<th>Dissociation constants $K_o$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E.N-MePLP</td>
</tr>
<tr>
<td>Succinate</td>
<td>9.8 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutarate</td>
<td>7.8 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adipate</td>
<td>21.0 ± 5.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>4.1 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>This work, estimated in 0.02 M triethanolamine hydrochloride, pH 7.2, 30°C.

<sup>b</sup>Fonda & Johnson (1970); estimated in 0.1 M triethanoloamine hydrochloride, pH 8.3, 25°C.

<sup>c</sup>Michuda & Martinez-Carrion (1970); estimated in 0.01 M TRIS-acetate, pH 8.2.

<sup>d</sup>Velick & Vavra (1962); 0.04 M arsenate, pH 7.4, 26°C.
as a first approximation, pK\textsubscript{3} for dicarboxylate complex is about 6.5. E.N-MePLP was titrated spectrophotometrically at pH 7.23 with several dicarboxylates. The data fitted well to equation (7) in METHODS (Figures 24-27). The values of K\textsubscript{O} obtained are summarized in Table 13.

**Reaction of E.N-MePLP with glutamate**

When glutamate is mixed with E.N-MePLP at pH 8.3 in 0.02M TEA•HCl, a biphasic reaction is observed. There is an instantaneous drop followed by a more gradual decrease of the absorption band at 376 nm (26.6 x 10\textsuperscript{3} cm\textsuperscript{-1}). Concomitantly, a new absorption band at 330 nm (30.0 x 10\textsuperscript{3} cm\textsuperscript{-1}) rises. An isosbestic point is observed at 348 nm (28.7 x 10\textsuperscript{3} cm\textsuperscript{-1}) and reaction finally comes to an equilibrium (Figure 28).

The absorption band at 330 nm possesses no circular dichroism suggesting that the coenzyme is released from the protein as N-MePMP. Indeed, after glutamate was allowed to equilibrate with 60 \muM E.N-MePLP, the absorption band at 330 nm could be removed by dialysis (Figure 29) or by gel filtration (data not shown).

Inclusion of 0.5 mM \alpha-ketoglutarate in the reaction mixture decreased the amount of the fast drop, retarded the slow phase and shifted the final equilibrium back towards the aldimine form of the enzyme (compare Figures 28 & 30). This indicates that glutamate is reversibly transaminated by E.N-MePLP to \alpha-ketoglutarate followed by the release of the amine form of the coenzyme.

**The effects of divalent anions on the reaction of E.N-MePLP with glutamate**

In the early stage of the investigation, the reaction of E.N-MePLP with glutamate was studied with E.N-MePLP prepared by combining one
Figure 28. Reaction of E.N-MePLP with 0.02 M glutamate at pH 8.3, 30 °C

E.N-MePLP (200 μM) was dialyzed exhaustively against 0.02 M triethanolamine hydrochloride, pH 8.3. The concentration of the enzyme was adjusted to 63 μM with the same buffer. The diluted enzyme (1.0 mL) was placed in a semicro cuvet. The reaction was initiated by mixing in with an addermixer 10 μL of 2 M L-glutamate. The numbers beside the curves indicate the time in minutes after the start of the reaction.
Figure 29. Release of N-MePMP

a) 1.0 mL of the apoenzyme of AAT (86 µM) in 0.02 M triethanolamine hydrochloride, pH 8.3, 25°C
b) One equivalent of N-MePLP was added to the apoenzyme in a)
c) The mixture in b) was equilibrated with 0.02 M glutamate
d) The solution in c) was dialyzed exhaustively against 0.02 M triethanolamine hydrochloride, pH 8.3 at 4°C
Figure 30. The effects of α-ketoglutarate on the reaction of E.N-MePLP with glutamate

The same reaction in Figure 28 was repeated in the presence of 0.5 mM α-ketoglutarate. The numbers indicate the time in minutes after the start of the reaction.
Figure 31. The effects of stoichiometric amount of phosphate on the reaction of E.N-MePLP with glutamate

The same reaction in Figure 28 was repeated in the presence of 60 \text{ \mu M} phosphate. The numbers indicate the time in minutes after the start of the reaction.
Figure 32. The effects of sulfate on the reaction of E.N-MePLP with glutamate

The same reaction in Figure 28 was repeated in the presence of 2 mM sulfate. The numbers indicate the time in minutes after the start of the reaction.
equivalent of N-MePLP with apoenzyme. Later, it was discovered that there was a difference in the rate of the slow phase as well as the poise of the final equilibrium depending upon whether or not the enzyme had been dialyzed subsequent to reconstitution. The dialyzed enzyme equilibrated more rapidly with glutamate although the final equilibrium lay considerably more to the side of the aldimine enzyme. However, if one equivalent of inorganic phosphate was added to the reaction mixture, the dialyzed E.N-MePLP reacted in a manner indistinguishable from that prepared by simply adding N-MePLP to the apoenzyme. Figure 28 shows the time course of a reaction between 60 μM E.N-MePLP and 0.02 M glutamate and Figure 31 shows the course of the same reaction in the presence of 60 μM phosphate. It turns out that sulfate can simulate the effect of phosphate although to a lesser extent. Figure 32 shows that 2 mM sulfate is required to achieve a result comparable to that with 60 μM phosphate.

These effects of dianion are readily understood since the apoenzyme binds one inorganic phosphate per active site with a dissociation constant of 1.6 x 10^{-6} M (Verge et al., 1979; Martinez-Carrion, 1975; Arnone, private comm., 1981). Both phosphate and sulfate have been used to displace PMP in the preparation of the apoenzyme (Wada & Snell, 1962; Scardi et al., 1963; Fonda & Auerback, 1976). Indeed, the apoenzyme used in the present study was prepared using phosphate. Upon reconstitution, all the bound phosphate is replaced by the 5'-phosphoryl group of N-MePLP. However, when the coenzyme is converted to N-MePMP, its covalent linkage with the protein is lost and its
affinity of the protein is weakened so that phosphate or sulfate can compete with it for binding to the active site.

Comparison between Figures 28, 31 and 32 shows that divalent anions have no effect of the initial fast drop. This fact together with the lack of circular dichroism at 330 nm suggests that the observed spectral changes when glutamate is reacted with E,N-MePLP may be described according to Scheme 11 of METHODS:

\[
\text{Glu} \xrightarrow{\text{kg}} \text{E}_L \xrightarrow{\text{N-MePMP}} \text{E}_M \xrightarrow{\text{SO}_4} \text{E} \leftrightarrow \text{E} \cdot \text{SO}_4
\]

When \( E_L \) and \( E_M \) represent \( E,N-\text{MePLP} \) and \( E,N-\text{MePMP} \); \( E \) and \( E \cdot \text{SO}_4 \) denotes the apoenzyme and its complex with sulfate. \( X \) represents all enzyme substrate intermediary complexes.

It is very tempting to assign the release of the coenzyme to the slow phase of the reaction. Thermodynamically, Scheme 11 may well be adequate. Perhaps, kinetically, the reaction is more complex. Analysis of the slow phase shows that it is a pseudo first order reaction in enzyme concentration for at least two half lives, and it is hard to see how this would be consistent with Scheme 11. In spite of this discrepancy, pseudo first order rate constants were obtained for the slow phase at several sulfate concentrations and when these were plotted against each other, a saturation curve resulted (Figure 33). It can be seen that the amount of sulfate for half saturation lies between 0.2 to 0.6 mM.
Figure 33. The effects of sulfate on the slow phase of the reaction of E.N-MePLP with glutamate at pH 8.3, 30°C

The reaction in Figure 32 was repeated in the presence of the indicated concentrations of K₂SO₄. For each point in the graph, the absorbance at 376 nm (26.7 x 10³ cm⁻¹) was monitored with respect to time. The pseudo first order rate constant, k₀, of the reaction was estimated from the half-life of the reaction according to the procedures described under Methods.
Estimation of the dissociation constants for glutamate ($K_5$) and α-ketoglutarate ($K_6$)

The reactions in consideration are only 4, 5 and 6 of Scheme 5 in METHODS. This portion of Scheme 5 is reproduced below:

![Diagram](attachment:image.png)

To estimate $K_5$ and $K_6$ is hardly a trivial problem. The spectrum of X has considerable absorption at 330 nm ($30.0 \times 10^3$ cm$^{-1}$), precisely where $E_M$ absorbs. The appearance of a band at 330 nm can mean the presence of X, $E_M$ or both. The corollary is that depending on the concentration of enzyme used in the experiment, the decrease of the absorption band for $E_L$ at about 370 nm caused by the addition of substrate can reflect mainly the extent of transamination rather than of complex formation.

X does have absorption elsewhere in the spectrum. In the reaction of native AAT with aspartate and particularly with erythro-β-hydroxy-L-aspartate, spectra of X are observed with good intensity at 430 nm ($23.3 \times 10^3$ cm$^{-1}$) and 492 nm ($20.3 \times 10^3$ cm$^{-1}$), respectively. However, for glutamate, complex mixture X of AAT absorbs weakly at 430 nm.

To simplify matters, $K_5$ can be considered first in the presence of a high concentration of α-ketoglutarate to block its dissociation from X. Unfortunately, this can give rise to the nonproductive complex $E_L.kg$. Thus, attempts were made to simultaneously solve for both $K_5$ and $K_6$. 
Application of Jenkins' methods

Jenkins and D'Ari (1966b) suggested titration at several constant levels of glutamate with enzyme. The idea is that at a given concentration of substrate, increasing the enzyme concentration favors complex formation which is detectable as a change in the apparent molar absorptivity. The mathematics involved are derived in Equations (9) and (10) in METHODS. In applying this method to the study of E.N-MePLP, because of the complication resulting from the release of N-MePMP, transamination is represented by the spectral change of the fast phase. The value of $A_{376}$ in Equation (10) was obtained from extrapolation of the time course of the slow phase to zero time. The data are presented in Figure 34. It can be seen that due to the long extrapolation involved to secure the values for the ordinate intercept, large errors are involved in the secondary plot (Figure 35). Despite this, this method provided a first approximation of the magnitude of the dissociation constants $K_5$ and $K_6$ as 1.0 M and 60 µM, respectively.

Saturation with N-MePMP

Attempts to estimate the dissociation constant for N-MePMP using circular dichroism by direct titration of the apoenzyme were unsuccessful. No signal was observed at 300 nm at 0.2 mM N-MePMP whereupon the spectrum became too noisy to be useful due to excessive absorption by the free coenzyme.

An empirical approach was adopted to establish the concentration of N-MePMP required for saturation. Reactions were run at one concentration of glutamate but with increasing concentrations of N-MePMP.

The slow phase was nearly eliminated at about 1 mM N-MePMP, suggesting
Figure 34. Application of Jenkins' method to the analysis of spectral data of the reaction of E.N-MePLP with glutamate at pH 8.3, 30°C

The procedures used were detailed under Methods. E.N-MePLP were previously exhaustively dialyzed in 0.02 M triethanolamine hydrochloride, pH 8.3.

A) The spectral data plotted according to equation 10 under Methods. The concentrations of enzyme are 40 μM (□), 78 μM (△) and 145 μM (○). The concentrations of glutamate are as indicated.

B) The plot of the inverse of the difference of the intercept in A) according to equation 9 under Methods.
Figure 34 (Continued)
Dialyzed E,N-MePLP (83 μM curve a) was equilibrated with glutamate (58 mM) in 0.02 M triethanolamine hydrochloride, pH 8.3, 30°C (curve b). Then N-MePMP was added to a final concentration of 59 μM (curve c) and of 177 μM (curve d).
Figure 36. Effects of α-ketoglutarate on the poise of the final equilibrium of a mixture of 83 μM E,N-MePLP, 0.46 M glutamate and 1.7 mM N-MePMP in 0.02 M triethanolamine hydrochloride, pH 8.3, 30°C

The added concentrations of α-ketoglutarate are indicated.
Figure 37. Effects of α-ketoglutarate on the poise of the final equilibrium of a mixture of 83 μM E.N-MePLP, 0.95 M glutamate and 1.7 mM N-MePMP in 0.02 M triethanolamine hydrochloride, pH 8.3, 30°C

The added concentrations of α-ketoglutarate are indicated.
Figure 38. Effects of α-ketoglutarate on the poise of the final equilibrium of a mixture of 83 μM E.N-MePLP, 1.86 M glutamate and 1.7 mM N-MePMP in 0.02 M triethanolamine hydrochloride, pH 8.3, 30°C

The added concentrations of α-ketoglutarate are indicated.
Figure 39. Estimation of the dissociation constants for glutamate ($K_g$) and for α-ketoglutarate ($K_o$)

The spectral data from Figures 36-38 were analyzed according to equations 11-14 by the procedures detailed under Methods. In this figure, the values of apparent molar absorptivity (ordinate) are plotted against the corrected concentrations of α-ketoglutarate (abscissa). The symbols '⋆' are experimental and '§' are calculated according to equation 11 using $K_g$ of 3.18 M and $K_o$ of 1.1. x $10^{-4}$ M.
that saturation had been achieved. Figure 36 shows the effect of N-MePMP on the final equilibrium of a mixture of glutamate and E.N-MePLP. Figures 37-39 show spectra of E.N-MePLP at several concentrations of glutamate and α-ketoglutarate in the presence of 1.7 mM N-MePMP. The absorbance at 330 nm is calculated to be about 16. Although absorption bands due to X were seen at 492 nm and 430 nm, they were too weak particularly at low concentrations of α-ketoglutarate. Therefore, the calculations for $K_5$ and $K_6$ were made at 377 nm ($26.5 \times 10^3$ cm$^{-1}$) according to Equations (11) to (14) in METHODS. Concentrations of α-ketoglutarate were chosen so that reaction 4 did not interfere. Since the interaction between glutamate and E is weak, reaction 7 is also insignificant.

The absorbances at 377 nm ($26.5 \times 10^3$ cm$^{-1}$) of Figures 37-39 were analyzed according to equation (11) by the NLIN program. It was necessary to hold $\varepsilon_X$ constant in the calculations. Runs were first made with different values, $\varepsilon_X$ ranging from 50 to 8000 at intervals of 1000. 3000 was found to give optimum fit between the calculated and experimental values of $A_{377}$. Next, adjustment was made of the concentration of α-ketoglutarate to include the amount generated in the course of the reaction. The procedure for doing this is detailed in METHODS.

After several iterations (Table 14), the values of $K_5$ and $K_6$ converged at 3.17 M and $1.10 \times 10^{-5}$ M. These values were refig into the program leaving $\varepsilon_X$ free as well. The final output from the NLIN are summarized in Table 15. Figure 40 compares the experimental data
<table>
<thead>
<tr>
<th>Figure</th>
<th>Conc'n of Glu (M) (x 10^{-3})</th>
<th>Conc'n (uM)</th>
<th>Conc'n (ug)</th>
<th>Conc'n (uM)</th>
<th>Conc'n (ug)</th>
<th>Conc'n (uM)</th>
<th>Conc'n (ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>1.86</td>
<td>6.56</td>
<td>0.640</td>
<td>7</td>
<td>746</td>
<td>0.609</td>
<td>0.363</td>
</tr>
<tr>
<td></td>
<td>5.53</td>
<td>0.538</td>
<td>0.227</td>
<td>20</td>
<td>266</td>
<td>0.512</td>
<td>0.305</td>
</tr>
<tr>
<td></td>
<td>5.29</td>
<td>0.514</td>
<td>0.217</td>
<td>22</td>
<td>219</td>
<td>0.489</td>
<td>0.291</td>
</tr>
<tr>
<td></td>
<td>4.98</td>
<td>0.483</td>
<td>0.204</td>
<td>26</td>
<td>174</td>
<td>0.459</td>
<td>0.274</td>
</tr>
<tr>
<td></td>
<td>4.66</td>
<td>0.450</td>
<td>0.191</td>
<td>30</td>
<td>128</td>
<td>0.430</td>
<td>0.256</td>
</tr>
<tr>
<td></td>
<td>4.25</td>
<td>0.412</td>
<td>0.174</td>
<td>34</td>
<td>84</td>
<td>0.392</td>
<td>0.234</td>
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<tr>
<td></td>
<td>3.91</td>
<td>0.378</td>
<td>0.164</td>
<td>38</td>
<td>63</td>
<td>0.360</td>
<td>0.214</td>
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<tr>
<td></td>
<td>3.62</td>
<td>0.349</td>
<td>0.147</td>
<td>42</td>
<td>42</td>
<td>0.332</td>
<td>0.198</td>
</tr>
<tr>
<td>38</td>
<td>0.95</td>
<td>7.38</td>
<td>0.768</td>
<td>6</td>
<td>252</td>
<td>0.747</td>
<td>0.227</td>
</tr>
<tr>
<td></td>
<td>7.05</td>
<td>0.733</td>
<td>0.158</td>
<td>9</td>
<td>206</td>
<td>0.713</td>
<td>0.217</td>
</tr>
<tr>
<td></td>
<td>6.69</td>
<td>0.695</td>
<td>0.150</td>
<td>13</td>
<td>161</td>
<td>0.676</td>
<td>0.206</td>
</tr>
<tr>
<td></td>
<td>6.20</td>
<td>0.643</td>
<td>0.139</td>
<td>18</td>
<td>117</td>
<td>0.626</td>
<td>0.191</td>
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<tr>
<td></td>
<td>5.60</td>
<td>0.581</td>
<td>0.125</td>
<td>24</td>
<td>74</td>
<td>0.565</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td>5.17</td>
<td>0.535</td>
<td>0.116</td>
<td>29</td>
<td>54</td>
<td>0.521</td>
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</tr>
<tr>
<td></td>
<td>4.74</td>
<td>0.489</td>
<td>0.106</td>
<td>34</td>
<td>34</td>
<td>0.476</td>
<td>0.145</td>
</tr>
</tbody>
</table>

Table 14. Successive approximation of $K_5^a$
The values of apparent molar absorptivity from Figures 37-39 together with the concentrations of glutamate as indicated in the table and those of α-ketoglutarate added in the reaction mixture were used to solve for the value of $K_5$ according to equation 11 under Methods. The first estimate of $K_5 = 4.4$ was employed to calculate the fractions of $E_L$ and $X$ and new concentrations of $E_M$ and $kg$. With the new values for $[kg]$, the second estimate of $K_5$ was obtained similarly to the first one. The fourth estimate of $K_5$ was 3.18 which was considered invariant with the third $K_5 = 3.17$ given in the table.
Table 15. Dissociation constant for glutamate ($K_g$) and α-ketoglutarate ($K_6$) found by the NLIN program

<table>
<thead>
<tr>
<th>Constant</th>
<th>Estimated values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_g$</td>
<td>$3.2 \pm 1.5 \text{ M}$</td>
</tr>
<tr>
<td>$K_6$</td>
<td>$1.1 \pm 0.5 \times 10^{-4} \text{ M}$</td>
</tr>
<tr>
<td>$\varepsilon_x$</td>
<td>$3.0 \pm 1.7 \times 10^3$</td>
</tr>
</tbody>
</table>
with those calculated. Thus, the equilibrium constant $K_e$ for the overall transamination reactions 5 and 6 can be calculated as follows:

$$K_e = \frac{[E_M][kg]}{[E_L][Glu]} = \frac{K_6}{K_3} = 3.5 \times 10^{-5}$$

The dissociation constant for N-MePMP ($K_g$)

Estimations of the value of $K_g$ were made according to equations (15) to (18) given in METHODS. The value of $K_e$ used was $3.5 \times 10^{-5}$ as determined above. The values for the absorbance at 376 nm ($A_{376}$) were taken from Figures 28, 30, and 36. Refinements of the estimated values of $K_g$ by the iterative procedure were done whenever necessary. The results are summarized in Table 16. It can be seen that except for the data taken from Figure 30, all others yielded a value of $K_g$ very close to the average of $5.1 \times 10^{-5}$ M. This is consistent with the observation that 1mM N-MePMP was required to block its dissociation.

There is no sound explanation as to why $K_g$ calculated using the data in Figure 30 was high, except that possibly α-ketoglutarate binds to the apoenzyme. This is not unreasonable since succinate has been found to bind the apoenzyme of AAT with a dissociation constant of 12 mM (Martinez-Carrion et al., 1973). If this is also true for α-ketoglutarate, the data in Figure 30 together with the value of $K_g$ at $5.1 \times 10^{-5}$ M, it can be calculated that the dissociation constant of the apoenzyme complex for α-ketoglutarate is $3 \times 10^{-5}$ M.

The dissociation constant for sulfate and phosphate ($K_{g'}$)

Estimation of the values of $K_{g'}$ for both dianions were made according to equations (20) to (23) given in METHODS. The values for $K_e$ and $K_g$
Table 16. Successive approximation of the dissociation constants for N-MePMP, $K_8$, and for divalent anions, $K_9$.

<table>
<thead>
<tr>
<th>Figure</th>
<th>$\varepsilon_L$ at 377 nm x 10^-3</th>
<th>Concentrations</th>
<th>Equation used</th>
<th>Diss. constant (uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$E_L$ (uM)</td>
<td>Glu(M)</td>
<td>$k_g$ (uM)</td>
</tr>
<tr>
<td>28</td>
<td>7.42</td>
<td>52</td>
<td>0.019</td>
<td>0.0</td>
</tr>
<tr>
<td>30</td>
<td>7.80</td>
<td>54</td>
<td>0.019</td>
<td>500</td>
</tr>
<tr>
<td>36</td>
<td>6.40</td>
<td>59</td>
<td>0.058</td>
<td>0.0</td>
</tr>
<tr>
<td>36</td>
<td>7.32</td>
<td>68</td>
<td>0.058</td>
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<tr>
<td>36</td>
<td>7.32</td>
<td>68</td>
<td>0.058</td>
<td>0.0</td>
</tr>
<tr>
<td>36</td>
<td>7.32</td>
<td>68</td>
<td>0.058</td>
<td>0.0</td>
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<td>36</td>
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<td>70</td>
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<td>0.0</td>
</tr>
<tr>
<td>36</td>
<td>7.54</td>
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<td>0.058</td>
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</tr>
<tr>
<td>36</td>
<td>7.54</td>
<td>70</td>
<td>0.058</td>
<td>0.0</td>
</tr>
<tr>
<td>31</td>
<td>4.51</td>
<td>31</td>
<td>0.019</td>
<td>0.0</td>
</tr>
<tr>
<td>31</td>
<td>4.51</td>
<td>31</td>
<td>0.019</td>
<td>0.0</td>
</tr>
<tr>
<td>32</td>
<td>5.61</td>
<td>31</td>
<td>0.019</td>
<td>0.0</td>
</tr>
<tr>
<td>32</td>
<td>5.61</td>
<td>31</td>
<td>0.019</td>
<td>0.0</td>
</tr>
<tr>
<td>33</td>
<td>5.09</td>
<td>31</td>
<td>0.055</td>
<td>0.0</td>
</tr>
<tr>
<td>33</td>
<td>5.09</td>
<td>31</td>
<td>0.055</td>
<td>0.0</td>
</tr>
<tr>
<td>33</td>
<td>2.91</td>
<td>18</td>
<td>0.055</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Values of $K_8$ and $K_9$ were calculated using the indicated equation from Methods together with the values of apparent molar absorptivity $\varepsilon_L$, and concentrations of the reacting species given in the indicated figures.
used were $3.5 \times 10^{-5}$ and $5.1 \times 10^{-5}$ M, respectively. The values for $A_{376}$ were obtained from Figures 31 and 32. In all cases reported, the concentration of E was found to be insignificant. Thus, $K_g$ was refined according to equations (22) and (23).

The results are summarized in Table 16. It can be seen that the average value of $K_g$ for sulfate is $1.5 \times 10^{-4}$ M. This is in good agreement with the kinetic data presented in Figure 33 in which the concentration of sulfate needed to decrease the rate of the slow phase by 50% is about 0.4 mM. The estimated value of $K_g$ for phosphate is $1.1 \times 10^{-6}$ M which is in agreement with the literature value of $1.6 \times 10^{-6}$ M (Verge et al., 1979).

Spectrum of X

Figure 40 shows a spectrum of the enzyme-substrate intermediary complex of E,N-MePLP with glutamate constructed using the dissociation constants found in this study and the spectrum of a mixture of 83 μM E.N-MePLP, 1.86 M glutamate and 0.75 mM α-ketoglutarate in Figure 39, which will be referred to as the "starting spectrum".

The "starting spectrum" is the sum of a mixture of E,N-MePLP and its complex with substrate. From Table 14, at 0.74 mM α-ketoglutarate, that this complex mixture consists of 61% $E_L$ and 39% X. A "1st Partial Spectrum of X" was calculated as follows:

1st Partial Spectrum of X = Starting Spectrum - 0.61 Spectrum of $E_L$ ÷ 0.39

Since the data in the "Starting Spectrum" terminated at 375 nm (28.0 x $10^3$ cm$^{-1}$), the rest of the spectrum was approximated as follows:
Step I: The "partial spectrum of X" was analyzed with four lognormal curves, Band I to IV. It was assumed that Bands I at 490 nm \( (20.4 \times 10^3 \text{ cm}^{-1}) \) and II at 465 nm \( (21.5 \times 10^3 \text{ cm}^{-1}) \) were due to the quinonoid intermediate; Band III at 430 nm was assumed to be due to a species that resembled the protonated form of E.N-MePLP. The height of I was compared to 40,000 which was assumed to be the molar absorptivity of Band I. The areas of Bands III and IV were compared to those of the protonated and deprotonated forms of E.N-MePLP (Figure 15). The fraction of the enzyme substrate complex absorbing at 330 nm \( (30.0 \times 10^3 \text{ cm}^{-1}) \) \( f_{330} \) was computed as follows:

\[
f_{330} = 1 - \left( \frac{\text{height of I}}{40,000} \right) - \left( \frac{\text{area of III}}{\text{area of protonated E}_L} \right) - \left( \frac{\text{area IV}}{\text{area of deprotonated E}_L} \right) = 1 - 0.03 - 0.56 - 0.4 = 0.2
\]

Step II: Since the "1st partial spectrum of X" only went up 357 nm, it must be extended to 300 nm \( (33.9 \times 10^3 \text{ cm}^{-1}) \) by summing the contribution of Bands III and IV from 357 nm \( (28.0 \times 10^3 \text{ cm}^{-1}) \) to 300 nm \( (33.0 \times 10^3 \text{ cm}^{-1}) \). This gives the "2nd partial spectrum of X."

Step III: The spectrum of the species absorbing at 330 nm was constructed as follows:

\[
S_{330} = (\text{Portion of apoenzyme spectrum from 375 nm to 300 nm}) + (f_{330})(\text{Portion of N-MePMP spectrum from 357 nm to 300 nm})
\]

Step IV: The final full spectrum of X (Final X) shown in Figure 40 was calculated as follows:

\[
\text{Final X} = (2\text{nd partial spectrum of X}) + (S_{330})
\]

The bands I to IV used to construct "Final X" are shown in Figure 40 together with three others, V to VII. The parameters for these are
Figure 40. The calculated spectrum of enzyme-substrate intermediary complex of E.N-MePLP in its reaction with glutamate at pH 8.3, 30°C
Table 17. Band shape parameters for the spectrum of the E,N-MePLP-glutamate complex (X) of Figure 40

<table>
<thead>
<tr>
<th>Peak position, $\nu_0$ (x 10^{-3} cm^{-1})</th>
<th>Peak height, $\varepsilon_0$ (x 10^{-3})</th>
<th>Width, W</th>
<th>Skewness, $\rho$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.4</td>
<td>490</td>
<td>1.13</td>
<td>1.10</td>
</tr>
<tr>
<td>21.5</td>
<td>465</td>
<td>0.50</td>
<td>1.30</td>
</tr>
<tr>
<td>23.3</td>
<td>429</td>
<td>4.24</td>
<td>3.90</td>
</tr>
<tr>
<td>25.0</td>
<td>400</td>
<td>0.25</td>
<td>3.90</td>
</tr>
<tr>
<td>26.7</td>
<td>375</td>
<td>1.73</td>
<td>3.90</td>
</tr>
<tr>
<td>29.9</td>
<td>334</td>
<td>2.55</td>
<td>3.40</td>
</tr>
<tr>
<td>35.8</td>
<td>279</td>
<td>30.00</td>
<td>4.76</td>
</tr>
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</table>


summarized in Table 17. Bands V and VI are for the bands at 330 nm and the protein absorption at 280 nm \((35.7 \times 10^3 \text{ cm}^{-1})\). Band VII was inserted to decrease the difference between the calculated and the experimental values at 400 nm. It was not entirely arbitrary since as it will be shown that in the reaction of E.N-MePLP with erythro-8-hydroxy-L-aspartate a band at 400 nm was observed.

**Activities if E.N-MePLP**

When Furbish et al. (1969) first prepared E.N-MePLP, they found that it had no activity when assayed in 0.0067 M aspartate and 0.0067 M \(\alpha\)-ketoglutarate in 0.067 M TEA.HCl pH 8.3. In contrast, the present study clearly indicates that E.N-MePLP is capable of reversible transamination. However, the dissociation constant for glutamate is very high. Thus, it seems that the cause of inactivity is at least partly due to the lack of substrate binding.

It was of interest to assay E.N-MePLP at a high aspartate concentration. On the other hand, it must be recognized that it was hard to prepare a sample of apoenzyme that is completely devoid of activity. It can only be kept minimal by successive treatments of the apoenzyme preparation with NaCNBH\(_3\) in the presence of \(\alpha\)-ketoglutarate.

In spite of these difficulties, Table 18 presents results of an attempt to assay the activity of E.N-MePLP. The procedures are detailed in METHODS. Although the activity of E.N-MePLP apparently goes up when the concentration of aspartate is at 1.8 M, the activity of the native enzyme actually goes down at 1.8 M aspartate as compared with that at 0.02 M aspartate. This could be the result of substrate inhibition.
Table 18. Activities of aspartate aminotransferase reconstituted with methylated analogs of PLP

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay mixture 1</th>
<th>Assay mixture 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific act. (µmol/s/mg)</td>
<td>Relative act. (%)</td>
</tr>
<tr>
<td>Native AAT</td>
<td>5.50 ± 0.28</td>
<td>100.0</td>
</tr>
<tr>
<td>E.PLP e</td>
<td>5.32 ± 0.28</td>
<td>95.0</td>
</tr>
<tr>
<td>E.N-MePLP a</td>
<td>0.020 ± 0.001</td>
<td>0.4</td>
</tr>
<tr>
<td>E.O-MePLP</td>
<td>0.0083 ± 0.0005</td>
<td>0.2</td>
</tr>
<tr>
<td>Eapo alone</td>
<td>0.0067 ± 0.003</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a The activity was assayed in terms of the production of oxalacetate at 30°C by coupling to malic dehydrogenase as described under Methods.

b 0.02 M aspartate, 0.02 M α-ketoglutarate, 0.1 M triethanolamine hydrochloride, pH 8.3.

c 1.8 M aspartate, 0.02 M α-ketoglutarate, 0.1 M triethanolamine hydrochloride, pH 8.3.

d The apoenzyme used was treated with NaCNBH₃ as described under Methods.

e The apoenzyme reconstituted with PLP.
due to the formation of nonproductive complex between aspartate and the pyridoxamine enzyme. The choice of 0.02 M $\alpha$-ketoglutarate might also have been too high for E.N-MePLP since $K_4$, the dissociation constant for $\alpha$-ketoglutarate from its nonproductive complex with E.N-MePLP, is 5 mM. Comparison of the activities needs to be at 1.8 M aspartate and at lower concentrations of $\alpha$-ketoglutarate.

**Reaction of E.N-MePLP with erythro-$\beta$-hydroxy-L-aspartate in 0.02 M TEA.HCl pH 8.3**

A biphasic reaction was observed when erythro-$\beta$-hydroxy-L-aspartate was mixed with E.N-MePLP. Although quite weak, an absorption band 492 nm (20 x 10$^{-3}$ cm$^{-1}$), characteristic of the quinonoid intermediate was observed in the spectrum. If the reaction was carried out at 4°C, the fast phase appeared not to be affected but the slow phase was very much retarded. Figures 41A and B show an experiment started at 4°C. Upon mixing 2 mM substrate with 50 $\mu$M E.N-MePLP, the band at 376 nm (26.7 x 10$^{-3}$ cm$^{-1}$) dropped immediately. In the resulting spectrum, besides the small band at 492 nm, there was a somewhat broadened band centered at 380 nm (26.4 x 10$^{-3}$ cm$^{-1}$). The system seemed to have come to equilibrium. It can be seen from curves b and d that there was little spectral change in about an hour during which time the circular dichroism (CD) spectrum of curve c was recorded. As the reaction mixture was allowed to warm to room temperature, weak negative CD bands at 400 nm (25.0 x 10$^{-3}$ cm$^{-1}$) and 330 nm (30.0 x 10$^{-3}$ cm$^{-1}$) developed (curves e and f). Correspondingly, the absorption band at 380 nm continued to decay giving rise to another one at 330 nm. Curiously enough, throughout
Figure 41. Reaction of E.N-MePLP with erythro-β-hydroxy-L-aspartate, pH 8.3

A) Absorption spectra and B) circular dichroism (CD) spectra. Curve a) 50 μM E.N-MePLP in 0.02 M triethanolamine hydrochloride, pH 8.3, 4°C. Curve b) 3 minutes after addition of hydroxyaspartate to a final concentration of 2 mM. Curve c) CD spectrum between 3 to 40 minutes after the start of the reaction. Curve d) Absorption spectrum at 50 minutes after the start of the reaction. After this spectrum was recorded, the reaction mixture was allowed to warm up in air to 25°C. Curves e) and f) are sequential scans taken between 10 to 35 minutes and 35 to 60 minutes, respectively, after standing at 25°C. Curve g) 67 minutes after standing at 25°C. C) Analysis of curve b) by resolution with lognormal curves as described under Methods. The symbols are experimental and the solid lines are calculated lognormal curves. D) The difference plot for C).
Figure 41 (Continued)
Figure 41 (Continued)
Table 19. Band shape parameters of curve b in Figure 41A

<table>
<thead>
<tr>
<th>Peak position, $v_0$ (x $10^{-3}$ cm$^{-1}$)</th>
<th>Peak height, $e_0$ (x $10^{-3}$)</th>
<th>Width, W (x $10^{-3}$ cm$^{-1}$)</th>
<th>Skewness, $\rho$</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.3</td>
<td>493</td>
<td>0.23</td>
<td>1.10</td>
<td>1.20</td>
</tr>
<tr>
<td>21.5</td>
<td>465</td>
<td>0.14</td>
<td>1.30</td>
<td>1.60</td>
</tr>
<tr>
<td>25.1</td>
<td>398</td>
<td>1.68</td>
<td>3.74</td>
<td>1.59</td>
</tr>
<tr>
<td>26.7</td>
<td>375</td>
<td>6.33</td>
<td>3.92</td>
<td>1.46</td>
</tr>
<tr>
<td>30.0</td>
<td>333</td>
<td>1.58</td>
<td>3.45</td>
<td>1.41</td>
</tr>
</tbody>
</table>
the entire experiment, the absorption band at 492 nm did not appear
to have changed any.

The width of the absorption band at 380 nm suggests that it was
a composite of overlapping bands. The spectrum at 3 min (curve 6)
was analyzed with lognormal curves, and as shown in Figure 42, a small
band could indeed be fitted at 400 nm (Table 19). The parameters for
the bands at 492 nm were adapted from those of native AAT.

**Reaction of E.N-MePLP with other amino acids**

Several other amino acids were tested with E.N-MePLP on 0.02 M
TEA.HCl, pH 8.3. These included cysteine sulfinate, aspartate, O-phos-
phoserine and alanine (Figures 42-45). The enzyme used was not
dialyzed after reconstitution and hence contained one equivalent of
inorganic phosphate. Qualitatively, these amino acids reacted in a
manner similar to glutamate in that they caused a decrease of the
absorption band at 376 nm (26.6 x 10³ cm⁻¹) and an increase of that
at 330 nm (30.0 x 10³ cm⁻¹). Further studies are needed to clarify
if these reactions are also biphasic. Also of interest are their
relative affinities for the enzyme. Nevertheless, it is interesting
to note that 3 mM cysteine sulfinate almost completely converted Eₐ₅₀
to products in about 10 min. This amino acid also reacts irreversibly
with the aldimine form of native AAT and converts it completely to
the amine form (Jenkins and D'Ari, 1966c).

**Spectra of crystalline E.N-MePLP**

Of all complexes of apoAAT-coenzyme analog, E.N-MePLP was the
first to be obtained in crystalline form. Single crystals of E.N-MePLP
can be grown at 4°C from 0.08 M sodium acetate buffer at pH 5.4
containing 8% polyethylene glycol (PEG) of molecular weight 6000. However, large plate-like single crystals suitable for study with polarized light absorption spectroscopy remain rare even from seeding with either a small crystal of native AAT or another one of E.N-MePLP. Protein crystals are in general fragile. Several good single crystals were obtained but they did not survive the mounting procedure. Therefore, crystal spectra of E.N-MePLP have only been obtained on two crystals. The spectra of one of these are shown in Figure 46. The other crystal, spectra of which were qualitatively identical to those shown in Figure 46, cracked before the instrumental conditions could be optimized. Diffraction studies are yet to be performed on the crystals of E.N-MePLP. Nonetheless, morphologically, the crystals of E.N-MePLP and those of native AAT are very similar. The assignment of the crystallographic axes in the case of E.N-MePLP were made solely by analogy to those of native AAT. Therefore, the spectra labelled $a$ and $c$ in Figure 46 were obtained with the plane of the polarized light beam parallel to the presumed $a$ and $c$ axes, respectively. The ratios of absorbances between the two spectra at 430 nm ($23.0 \times 10^3$ cm$^{-1}$) and 376 nm ($26.6 \times 10^3$ cm$^{-1}$) are 0.30 and 0.37, respectively. The corresponding ratios for native AAT are 0.67 and 0.5. If the assignment of the crystallographic axes in the crystals of E.N-MePLP were indeed correct, the difference in the ratios between the spectra observed on E.N-MePLP can be interpreted as being due to change in the orientation in which the methylated coenzyme is bound in the protein as compared with that in native AAT (Metzler et al., 1978).
The reaction was performed in 0.02 M triethanolamine hydrochloride, pH 8.3 (1.0 mL) at room temperature in the sample compartment of a Cary 1501 spectrophotometer. Addition of the amino acid initiated the reaction. The numbers beside the curves indicate the time in minutes after the start of the reaction.
Figure 43. Reaction of E.N-MePLP (68 μM) with aspartate (14 mM) at pH 8.3

The reaction was performed similarly to that shown in Figure 42. The numbers beside the curves indicate the time in minutes after the start of the reaction.
Figure 44. Reaction of E.N-MePLP (55 µM) with alanine (30 mM) at pH 8.3

The reaction was performed similarly to that shown in Figure 42. The numbers beside the curves indicate the time in minutes after the start of the reaction.
Figure 45. Reaction of E.N-MePLP (55 μM) with O-phosphoserine (30 mM) at pH 8.3.

The reaction was performed similarly to that shown in Figure 42. The numbers beside the curves indicate the time in minutes after the start of the reaction.
Figure 46. Spectra of a single crystal of E.N-MePLP

The crystal was grown from 0.08 M sodium acetate buffer, pH 5.4, containing 8% polyethylene glycol, molecular weight 6000, as described under Methods. Curve a) The spectrum of c-polarization. Curve b) The spectrum of a-polarization.
The image contains a graph with the following details:

- **Absorbance** is plotted on the y-axis, ranging from 0.00 to 1.20.
- **WAVE NO. (X10^-3/CM)** is plotted on the x-axis, ranging from 16.00 to 35.00.

There are two peaks labeled 'a' and 'b' on the graph.
Unlike other aldehyde analogs of vitamin B₆ phosphate, O-MePLP has no absorption in the visible region of the spectrum. At pH 8, the spectrum of O-MePLP shows two bands in the ultraviolet, one at 333 nm (30.0 x 10^3 cm⁻¹) and the other at 280 nm (35.8 x 10^3 cm⁻¹) (Figure 47). When the apoenzyme of aspartate aminotransferase is mixed with one equivalent of O-MePLP at 25° C, no distinct absorption band is observed. However, at 333 nm a shoulder appears on the side of the protein absorption band (Figure 48). Addition of excess coenzyme analog only increases absorbance at 333 nm.

At pH 8, addition of either L-aspartate (Figure 49), L-glutamate (Figure 50) or erythro-β-hydroxy-L-aspartate (Figure 51) the shape of which is characteristic of the quinonoid intermediate. This phenomenon proved to be enzymatic since no reaction was observed when the apoenzyme was excluded from the mixture. Three other amino acids, cysteine sulfinate, O-phosphoserine and 2-amino-3-phosphonopropionate, were tested. None of these at 20 mM caused any spectral change with the apoenzyme-analog mixture (data not shown). At 30° C, the intermediate absorbing at 480 nm is transient. It is formed rapidly and attains a maximum in less than a minute. Then it decays more slowly with a half-life of minutes. The reaction of E.O-MePLP with aspartate was investigated in more detail.

The reaction of E.O-MePLP with aspartate at 30° C

The plot of $k_0$, the observed rate constant for the decay of the quinonoid intermediate at 30° C, against pH (Figure 52) was found to
Figure 47. The spectrum of O-MePLP in a solution of 0.02 M triethanolamine hydrochloride, pH 8.3, 25°C
Figure 48. The spectrum of E.0-MePLP in a solution of 0.02 M triethanolamine hydrochloride, pH 8.3, 25°C

The spectrum of the apoenzyme has been subtracted.
Figure 49. Reaction of L-aspartate with the apoenzyme of AAT in the presence of excess 0-MePLP

A 1.0 mL solution of apoenzyme (70 µM) in 0.02 M triethanolamine hydrochloride, pH 8.3 was placed in a cuvet held in the sample compartment of the spectrophotometer (a). To this solution, 0-MePLP was added first to a final concentration of 70 µM (b) and then 200 µM (c). After this, aliquots of 1.0 M L-aspartate were added. Each spectrum was recorded when the absorbance at 481 nm (20.8 x 10^3 cm⁻¹) attained a maximum. The final concentrations of aspartate are as indicated beside the curves in the figure.
Figure 50. Reaction of L-glutamate with 70 μM apoAAT in the presence of three equivalents of O-MePLP and 0.02 M triethanolamine hydrochloride, pH 8.3, 25°C

The final concentrations of glutamate are as indicated beside the curves. The spectra were recorded after the band at 481 nm (20.8 x 10^3 cm^{-1}) had attained a maximum.
Figure 51. Reaction of erythro-β-hydroxy-L-aspartate with 70 μM apoAAT in the presence of one equivalent of O-MePLP and 0.02 M threethanolamine hydrochloride, pH 8.3, 25°C.

The final concentrations of hydroxyaspartate are as indicated beside the curves. The spectra were recorded after the band at 481 nm (20.8 x 10^3 cm^{-1}) had attained a maximum.
Figure 52. Variation of the apparent rate constant, $k$, for the decay of the quinonoid intermediate at 30°C as a function of pH.

The values of $k$ were estimated as described under Methods. The symbols '★' are experimental while '♯' are calculated according to equation 27 under Methods using values for $pK_o = 8.14$, $k_A = 0.027 \text{ s}^{-1}$ and $k_B = 0.004 \text{ s}^{-1}$. 
be described by equation (26) in METHODS with $k_A = 0.027 \pm 0.001 \text{ s}^{-1}$, $k_B = 0.004 \pm 0.0005 \text{ s}^{-1}$ and $-\log_{10} K = 8.14 \pm 0.07$. This means that the decay reaction for the quinonoid intermediate can be depicted minimally by Scheme 13 in METHODS which is reproduced below:

\[
\begin{array}{c}
\text{Q} \\
\text{HQ} \\
\text{P}
\end{array}
\begin{array}{c}
\text{K}_O \\
\text{K}_A \\
\text{K}_B
\end{array}
\]

Q is the quinonoid intermediate while HQ is a colorless species with no absorption in the visible region of the spectrum. Q can be reversibly protonated with the dissociation constant $K$, which $pK_a$ is 8.14. Thus, there are two pathways to products. At high pH, the reaction with rate constants $k_B = 0.004 \text{ s}^{-1}$ predominates while at low pH, that with $k_A = 0.027 \text{ s}^{-1}$ is preferred.

The question arises as to what the decay product of Q is. The experiment summarized in Figure 53 shows that oxaloacetate, as assayed by MDH, is produced at the same rate as Q decays. In other words, E.O-MePLP is capable of transaminating aspartate to oxaloacetate. It appears that O-MePMP is also released from the protein matrix. Figure 54 shows that addition of $5 \times 10^{-7} \text{ M} \text{ O-MePMP}$ has no effect on the decay reaction of Q. However, $1.3 \times 10^{-5} \text{ M} \text{ O-MePLP}$ does decrease the rate of decay of Q by about one-half. Furthermore, when the decay reaction of Q is completed, addition of O-MePLP will cause an increase of absorbance at 480 nm (data not included). This suggests that at the end of the decay of Q, the coenzyme binding site on the protein is
Figure 53. Time course for the reaction of E.O-MePLP with L-aspartate at 30°C

Each reaction mixture contained 1.9 M aspartate, 8 units of malic dehydrogenase, 0.1 mM NADH in a total volume of 1.0 mL and at the indicated pH. The reaction was initiated by addition of enzyme. The open symbols trace the change of absorbance at 480 nm (20.8 x 10^3 cm^-1). The closed symbols trace the change of absorbance at 340 nm (29.4 x 10^3 cm^-1) at 10 times the actual values observed.
Figure 54. Effects of an excess of O-methylated coenzyme analogs on the reaction of E.-O-MePLP with L-aspartate at 30°C

Each reaction was initiated by addition of the enzyme to a final concentration of 13 μM. The reaction mixture contained 0.2 M aspartate alone (o) or together with either 49 μM O-MePMP (+), or 13 μM O-MePLP, (Δ), or 38 μM O-MePLP (α).
Figure 55. Time course of a double pH-jump experiment at 30°C

The reaction of E.O–MePLP (24 μM) and L-aspartate (2.0 M) was initiated at pH 8.5. The pH of the mixture was maintained at pH 8.5 (o), or jumped to 6.1 at 20 s (O), or jumped to 6.1 at 20 s and rejumeped once more to 8.5 at 40 s (Δ).
Figure 56. Time course of a single pH-jump experiment at 30°C

The reaction E.0-MePLP (24 μM) and L-aspartate (2.0 M) was run at pH 8.5 (o). The same reaction initiated at pH 5.4 and jumped to pH 8.5 at 40 s (C), 80 s (Δ) and 120 s (O).
mostly vacant. No further reaction takes place unless the protein is replenished with O-MePLP.

At a pH below 7 no absorbance is seen at 480 nm. The reaction, nevertheless, proceeds. This was demonstrated by the pH jump experiment summarized in Figure 55. When the reaction was started at pH 8, the intermediate at 480 nm was seen to rise rapidly to a maximum in 20 s. At this point, the pH was suddenly lowered at 6; no absorbance could be detected at 480 nm. At 50 s, the pH was jumped back to 8; the intermediate returned. A curious fact is that absorbance at 480 nm reappeared and attained a maximum in 20 s, but it did not attain the same intensity as it had before the pH was jumped down.

When the reaction was started at pH 7, initially no absorbance at 480 nm was seen. If at any time, the pH was raised abruptly to 8, the absorbance at 480 nm appeared. Figure 56 shows the results of a set of experiments in which each reaction was started at pH 5.3 but jumped to pH 8 at different times. This generated a family of curves of very similar shapes. Notice that the later the upward pH jump, the lower the maximum intensity attained.

The reaction of E.O-MePLP with aspartate at 5.5° C

At 5.5° C, the intermediate absorbing at 480 nm is stable for minutes depending on the concentration of substrate present. Figure 57A shows how the apparent extinction coefficient at 480 nm changes as a function of aspartate at 5 different pH values at 55° C. Notice that the shape of all the curves is similar except for that of pH 9.3. The same set of data is represented in a Scatchard plot in terms of
aspartate in Figure 57B. The pronounced curvature indicates that the system is more complex than simple \( E + S \rightleftharpoons [Q + HQ] \). In spite of this except for pH 9.3 the shape of the curves is extraordinarily similar suggesting that the affinity of E.O-MePLP for aspartate is pH-independent. When the data in Figure 57B were replotted in terms of the concentration of protons, another family of curves resulted as seen in Figure 57C. The lines at low concentrations of aspartate are not linear but the line for 1.75 M aspartate appears to be so. The data for the variation with pH of apparent molar absorptivity at 480 nm generated with 1.75 M aspartate fitted very well to equation (25) with a \( pK_a = 7.7 \pm 0.04 \) (Figure 57D). A spectrum of E.O-MePLP in a 2 M aspartate solution at pH 9.3 is shown in Figure 58.
A) Variation of the values of apparent molar absorptivity at 480 nm (20.8 x 10³ cm⁻¹) with different concentrations of aspartate at the indicated pH. B) Scatchard plot of the data in A) with respect to the concentrations of aspartate. The dashed lines tie together the data points obtained with the indicated concentrations of aspartate at the following values of pH: 7.4 (o), 7.7 (Δ), 8.0 ( ), 8.3 (o) and 9.3 (Δ). C) Scatchard plot of the data in A) with respect to proton concentrations. The dashed lines tie together the points obtained at the indicated pH. D) The data for 1.75 M aspartate are fitted to equation (25) under Methods using a pK₀ of 7.7. The symbols '§' are experimental while '*' are calculated according to equation 25 under Methods using values for pK₀ = 7.7.
Figure 57 (Continued)
Figure 57 (Continued)
Figure 58. The spectrum of E.O-MePLP in the presence of 2.0 M L-aspartate at pH 9.3, 5.5°C
DISCUSSION

Spectra of N-MePLP and its Schiff base with valine

It can be seen in Tables 5 & 7, that the positions and band shapes of major absorption bands for PLP and N-MePLP and their Schiff base are within 5% of each other. The molar absorptivities of the bands for the N-methylated analog do tend to be higher than those of PLP. There are larger differences in the values of width and skewness of the minor or "buried" bands. However, these are not to be emphasized since the parameters of these minor bands were preselected and held constant during the computer-assisted analysis.

In view of the structural similarities between PLP and N-MePLP, it is not expected that their spectral properties would be very different. The analysis reported in this thesis does confirm that the effect of N-methylation is indistinguishable from that of N-protonation.

Dissociation constants for N-MePLP and its Schiff base with valine

The values of pK for N-MePLP and for PLP are very similar (see Scheme 14). In contrast, the values of pK for proton dissociation from the imino nitrogen of the two Schiff bases are separated by 2 units (Table 9). The difference reflects the effects of the presence of a positive charge at the ring nitrogen on proton dissociation at the imino nitrogen. The corresponding pK for the Schiff base of N-methyl-5-deoxy-pyridoxal is 9.80 while that of all other imines of vitamin B6 related aldehydes are about 12 (Metzler et al., 1980). The high pK value has long been attributed to a chelating effect on the hydrogen as is indicated in the structures below:
It has been well established that in Schiff bases of salicylaldehyde with 2-aminopropane, the tautomer \( \text{II} \) is favored in aqueous solution (Heinert & Martell, 1963; Herscovitch et al., 1973; Inouye, 1967). As is shown above are three resonance forms of \( \text{II} \) (a to c). Extending this concept to Schiff bases of vitamin B\(_6\) aldehydes, it can be seen that if the ring nitrogen is positively charged, charge pairing can stabilize a negative charge at the 3- and 5-positions of the ring. Thus N-methylation or N-protonation can result in sequestering the negative charge of the 3-phenolic oxygen into the ring rather than having it delocalized towards the imino nitrogen. This will lead to the observed decrease in the value of pK for the imino nitrogen.

The formation constants for Schiff bases reported vary considerably from one compound to the next (Metzler et al., 1980). The values for the logarithm of the formation constant for the valyl Schiff bases of the N-protonated ionic form of 5'-deoxypyridoxal and its corresponding N-methylated analogs are 1.82 and 2.06 (Metzler et al., 1980). This trend is not observed in the Schiff bases of PLP and N-MePLP, which are 1.84 and 1.58. The effect of the positive charge on the ring nitrogen is seen in the pH dependence of the value of \( K_{\text{PH}} \), the concentration ratio:

\[
\frac{[\text{Schiff base} \text{ total}]}{[\text{amine} \text{ total}]} \times [\text{aldehyde} \text{ total}] 
\]

For N-MePLP, \( K_{\text{PH}} \)
is invariant with pH in the range 6 to 10 (Figure 10). However, for other vitamin B<sub>6</sub> analogs, $K_{pH}$ changes about two fold when the pH increases from 7 to 8 as a result of the deprotonation of the ring nitrogen.

**0-MePLP and its Schiff base**

Unlike N-methylation, which results in little change in the absorption spectrum of PLP in aqueous solution, 0-methylation of the 3-phenolic oxygen shifts the spectrum of the coenzyme chromophore entirely into the ultraviolet region. Although in this thesis no detailed study of the acid dissociation and equilibria of Schiff base formation is reported for O-MePLP, considerable information is available in the literature.

Metzler et al. (1973) and Harris et al. (1976) have compared a large number of derivatives of 3-hydroxypyridine and found that they all have two apparent pKs at about 4 and 8. These are assignable to the ionization of the 3-phenolic oxygen and the ring nitrogen, respectively. However, 0-methylated 3-hydroxypyridine has only one pK at 4.88. Metzler and Snell (1955) determined the pK value for 0-methylpyridoxal as 4.75. Pocker and Fischer (1969) reported a single pK for O-MePLP as 4.15. Thus, it can be seen that other than blocking protonation at the 3-phenolic oxygen, 0-methylation also shifts the pK of the ring nitrogen of the coenzyme to about 4.

Feldmann and Helmreich (1976) recorded spectra of both O-MePLP and its Schiff base with an n-alkylamine in dimethylsulfoxide solution and found that their absorption bands peak at 295 nm ($33.9 \times 10^3 \text{ cm}^{-1}$) and 278 nm ($36.0 \times 10^3 \text{ cm}^{-1}$), respectively. Hence, it is understandable
that bound O-MePLP only exhibits as a shoulder of the protein absorption band at 330 nm (30.0 x 10^3 cm^{-1}, Figure 48).

Of particular interest is the value of pK for the imino nitrogen of a Schiff base of O-MePLP. Herscovitch et al. (1973) have estimated that for the isopropylamino Schiff base of para-nitrosalicylaldehyde, the two apparent pK values are about 2.93 and 9.9 corresponding to the following:

\[
\begin{align*}
\text{iii} & \quad \text{iv} \\
\text{v} &
\end{align*}
\]

By analogy, for a Schiff base of O-MePLP, the value of pK for the ionization of the proton of the imino nitrogen would be lower than 3:

\[
\begin{align*}
\text{vi} & \quad \text{vii}
\end{align*}
\]

While the effects of various ring substituents on the pK value of the imino nitrogen are well studied, much less is reported on the pK value of the ring nitrogen. Therefore, it is hard to predict whether
Protonation occurs at the ring nitrogen prior to, or subsequent to, that of the imino nitrogen. Nevertheless, as a first approximation, the value of the pK for the ring nitrogen in a Schiff base of O-MePLP should not differ widely from that of O-MePLP, which is 4.18.

Proton dissociation from the imino nitrogen of the bound N-MePLP

As pointed out under Literature Review, for native AAT, the value of pK\textsubscript{1} is 5.3, the values of pK\textsubscript{4} for the monovalent anion and for the dicarboxylate complexes are about 7 and 9. By contrast, in E.N-MePLP, the value of pK\textsubscript{1} is estimated to be 5.1, the values of pK\textsubscript{4} for the two kinds of anion complexes are 6 and 6.5, respectively.

It should be clear from the foregoing discussion on proton dissociation from Schiff bases of the methylated analogs of PLP that the pK for a proton chelated between the imino nitrogen and the 3-phenolic group as in structure viii is about 12. The presence of a positive charge at the N-1 position as in ix lowers this by 2.2 units through the charge-pairing mechanism analogous to that described for ii. O-protonation should have similar effect as O-methylation in diminishing the chelating effect of the 3-phenolic oxygen and resulting in a shift of the pK to below 3 as in structure x.

In native AAT, a network of hydrogen bonds from various side chains of the protein to different positions on the bound coenzyme can alter the electronic structure of the latter and vary the pK at the imino nitrogen. Conceivably, the negative charge of the 3-phenolic oxygen of the coenzyme may be neutralized by the positive charge of the guanidinium group of the
\[ pK \text{ about } 12 \]

\[ \text{viii} \]

\[ pK = 9.6 \]

\[ \text{ix} \]

\[ pK \text{ below } 3 \]

\[ \text{x} \]

\[ pK \text{ about } 5.4 \]

\[ \text{xi} \]

\[ pK \text{ about } 7 \]

\[ \text{xii} \]
side chain of Arg-385 through hydrogen bonding via the amide grouping of Asn-194 (structure xi). Since the carboxylate group of Asp-222 is interacting with the pyridinium nitrogen, the coenzyme probably only carries a partial positive charge. The situation is intermediate between those of structures ix and x. It is not surprising that $pK_a$ of native AAT has a value as low as 5.3.

Binding of a monovalent anion to Arg-386 can interrupt the hydrogen bond between Asn-194 and Arg-386 as is shown in xii. The negative charge on the 3-phenolic is not neutralized. However, due to the remaining hydrogen bond with Tyr-225, the chelating effect of the 3-phenolic oxygen on the proton at the imino nitrogen is moderated. Hence, the $pK_a$ value for the enzyme complex with a monovalent anion is about 7.

In the enzyme dicarboxylate complex, the carboxyl groups of the inhibitor bind to Arg-386 and Arg-292. The carbon chain extends across the central portion of the coenzyme ring. In native AAT, this may cause the coenzyme ring to move slightly closer to Asp-222 leading to a stronger interaction between the ring nitrogen and the carboxyl group of Asp-222. The positive charge of the ring is thereby neutralized resulting in an increase of the value of $pK_a$ to 9. However, steric crowding of the N-methyl group prevents such movement of the coenzyme ring and the value of $pK_a$ for a dicarboxylate complex of E.N-MePLP remains only at 6.5.

The state of protonation of bound O-MePLP

For native AAT and E.N-MePLP, the distinct spectra of the bound coenzyme make it possible to obtain precise data on proton dissociation
from the imino nitrogen. The variation in the observed values of the pK as compared with those of the free coenzymes can be rationalized in terms of hydrogen bonding to the side chains of amino acids of the protein.

For E.0-MePLP, the spectrum of the bound coenzyme is obscured by the absorption band of the protein. However, the shoulder visible at 330 nm \((30.0 \times 10^3 \text{ cm}^{-1})\) suggests that the band due to bound O-MePLP should peak at about 295 nm similar to its free Schiff base in dimethylsulfoxide as reported by Feldmann and Helmreich (1976). This means that the imino nitrogen of bound O-MePLP is deprotonated at pH 8, as protonation should shift the absorption band to a wavelength longer than that of O-MePLP. This is consistent with the fact that the imino nitrogen of a free Schiff base of O-MePLP should be lower than 3, as discussed already.

It is hard to tell from the spectrum of E.0-MePLP whether the ring nitrogen is protonated or not, since protonation is not expected to influence the position of the absorption band. However, in view of the fact that the reaction of aspartate with E.0-MePLP does produce the quinonoid intermediate, a process that is facilitated by a positively charged coenzyme ring acting as electron sink, it is likely that N-1 in E.0-MePLP is protonated and hydrogen bonded to the carboxylate group of the side chain of Asp-222 even at pH 9.

**Spectra of E.N-MePLP**

The spectral properties of E.N-MePLP in solutions resemble those of native AAT (Table 10). The values of \(\Delta A/A\) for the circular dichroism of the bound coenzymes for both enzymes are identical. The molar absorpti-
vities are higher for E.N-MePLP than for native AAT which correlates with the higher values for N-MePLP than of PLP. For the absorption band of protonated E.N-MePLP at 430 nm \((23.3 \times 10^3 \text{ cm}^{-1})\), the difference plot displays a pattern similar to that found for native AAT and for glutamate decarboxylase (Metzler et al., 1980). However, the skewness of the band for E.N-MePLP is about 0.1 greater than for native AAT. This is not considered significant since band parameters obtained from analyses of different preparations of native AAT involve an uncertainty of \(\pm 0.1\). This variation can be attributed to the difference in low amounts of colored contaminants in the preparations. The presence of these contaminants can also account for the misfit of the lognormal curve at 400 nm \((25.0 \times 10^3 \text{ cm}^{-1})\) in the spectrum of the deprotonated form of E.N-MePLP (Figure 11).

For the spectra of crystalline E.N-MePLP, a change in the linear dichroism is observed. This change is seen in the polarization ratio, the ratio of the absorbance in the \(\alpha\)-polarization relative to that in the \(\gamma\)-polarization. This ratio is 0.33 in E.N-MePLP in contrast to 0.66 for native AAT (Metzler et al., 1978). Disparities in the polarization ratio have been interpreted to reflect conformational alterations of the bound coenzyme ring. Analogously, the orientation of bound N-MePLP can be interpreted as being different from that of PLP. The crystals of E.N-MePLP are probably isomorphous with those of native AAT since those of E.N-MePLP can be grown from a seed crystal of native AAT. Considerable difficulty was encountered in the attempt to collect spectroscopic as well as x-ray
diffraction data. The crystals of E.N-MePLP are unstable and crack easily upon warming to room temperature. In addition, there is the complication that the two subunits of native AAT are not exactly symmetrically disposed with respect to the diad axes in the crystal (Arnone, private comm., 1981). Therefore, these data from the spectral studies of crystalline E.N-MePLP are not interpreted quantitatively at present.

**Ligand binding of E.N-MePLP**

Under Literature Review, it has already been pointed out, that because AAT has affinity for many anions, the binding of ligands is actually an ion exchange process. Therefore, when comparing the affinities of the protein for any ligand, attention must be paid to the amounts and the identities of other anions present in solution.

In this study, the dissociation constants for chloride were obtained in acetate buffer which only had little effect on the proton dissociation from E.N-MePLP. Jenkins (1980) studied binding of chloride to AAT in cacodylate buffer, a buffer which was shown to have insignificant effect. Despite this, the magnitudes of the dissociation constants of chloride for both proteins are very similar (Table 12).

Turning to the binding of dicarboxylates, literature values of dissociation constants for their complexes with AAT were estimated for different buffers and at various pH. Nevertheless, the relative magnitude of these are similar and they follow the sequence $\alpha$-ketoglutarate < glutarate < adipate < succinate. The corresponding values obtained for E.N-MePLP are of similar order of magnitude except that the sequence of
adipate was altered; instead, in this case it is α-ketoglutarate < glutarate < succinate < adipate (Table 13).

Small anions and dicarboxylates seem to react with E,N-MePLP in almost the same way as they do with native AAT. However, a large difference is seen in the value of $K_5$, the dissociation constant for glutamate. Between pH 7 and 8, a substrate like glutamate has a sum of one negative charge while a dicarboxylate like glutarate has two. In the estimation of $K_5$, the dissociation constant for glutamate, the reaction being observed is:

$$E_L + \text{O}_2\text{C(CH}_2\text{)}_2\text{CHCNH}^+\text{C}_2\text{O}_2^- \rightleftharpoons K_5 \rightleftharpoons X$$

For glutarate, however, $K_6$, the dissociation constant observed is actually that of a two-step reaction involving an uptake of a proton:

$$E_L + \text{O}_2\text{C(CH}_2\text{)}_3\text{C}_2\text{O}_2^- \rightleftharpoons K_4 \rightleftharpoons E_L . \text{O}_2\text{C(CH}_2\text{)}_3\text{C}_2\text{O}_2^-$$

Therefore in order to account for the effect of the difference in charge, the following reaction for glutarate can be considered:

$$E_L + \text{O}_2\text{C(CH}_2\text{)}_3\text{C}_2\text{O}_2^- \rightleftharpoons K_5' \rightleftharpoons H^+ E_L . \text{O}_2\text{C(CH}_2\text{)}_3\text{C}_2\text{O}_2^-$$
$K_o'$ can be related to $K_o$ using the acid dissociation constant $K_a$ for glutarate as follows:

\[
K_a = \frac{[H][\text{O}_2C(\text{CH}_2)_3\text{CO}_2^-]}{[\text{HO}_2C(\text{CH}_2)_3\text{CO}_2^-]}
\]

\[
K_o = \frac{[E_L][\text{O}_2C(\text{CH}_2)_3\text{CO}_2^-]}{[H^+E_L^-\text{O}_2C(\text{CH}_2)_3\text{CO}_2^-]}
\]

\[
K_o' = \frac{[E_L][\text{HO}_2C(\text{CH}_2)_3\text{CO}_2^-]}{[H^+E_L^-\text{O}_2C(\text{CH}_2)_3\text{CO}_2^-]}
\]

\[
= \frac{K_o [H^+]}{K_a}
\]

Table 20 compares the above constants for E,N-MePLP and native AAT. It is seen that N-methylation of the coenzyme affects the binding of glutarate little but it decreases the affinity of the protein for glutamate by almost four orders of magnitude. This difference may be related to the fact the enzyme and a dicarboxylate are held together via ionic linkages while the complex of enzyme and substrate involves covalent bonds.

Both a dicarboxylate and a substrate are attracted to the active site by electrostatic forces. A dicarboxylate can clearly adapt very
<table>
<thead>
<tr>
<th>Definition of constant</th>
<th>Native AAT(^a)</th>
<th>E.N-MePLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_o) = (\frac{[E_L][\text{O}_2\text{C(CH}_2)_3\text{CO}_2^-]}{[\text{H.E}_L\cdot\text{O}_2\text{C(CH}_2)_3\text{CO}_2^-]})</td>
<td>(3 \times 10^{-3}) M (pH 7.4)</td>
<td>(4.1 \times 10^{-3}) M (pH 7.2)</td>
</tr>
<tr>
<td>(K_o' = \frac{[E_L][\text{HO}_2\text{C(CH}_3\text{CO}_2^-]}}{[\text{H.E}_L\cdot\text{O}_2\text{C(CH}_2)_3\text{CO}_2^-]})</td>
<td>(3.1 \times 10^{-5}) M</td>
<td>(6.8 \times 10^{-5}) M</td>
</tr>
<tr>
<td>(K_5 = \frac{[E_L][\text{O}_2\text{C(CH}_2)_2\text{CH(NH}_3^+\text{CO}_2^-]}{[\text{H.E}_L\cdot\text{O}_2\text{C(CH}_2)_2\text{CH(NH}_3\text{CO}_2^-]})</td>
<td>(9 \times 10^{-4}) M</td>
<td>(3.4) M</td>
</tr>
<tr>
<td>(\frac{K_5}{K_o'})</td>
<td>29</td>
<td>(5 \times 10^4)</td>
</tr>
</tbody>
</table>

\(^a\) Velick & Vavra (1962)

\(^b\) \(K_a = 3.8 \times 10^{-6}\) M from Handbook of Biochemistry (1968).
well both sterically and electronically to the binding site and therefore forms a tight complex with the enzyme. On the other hand, the substrate, being sterically and electronically different, probably does not bind tightly unless a covalent bond is formed. Therefore, the difference between native AAT and E.N-MePLP in this regard is that somehow, the formation of the covalent complex in the latter is impeded. Two factors that can contribute to this may be operative simultaneously.

Because the protonated α-amino group is not a nucleophile, proton transfer must take place between α-amino group and the imino nitrogen before addition of the amino group to the 4'-carbon of the bound coenzyme. The pK of the proton of the α-amino group has a value of 9.6. The imino nitrogen of the bound coenzyme in the enzyme substrate complex is assumed to be 9 which is similar to that in the enzyme-dicarboxylate complex. Under these conditions, proton exchange can take place. However, this is not readily possible for E.N-MePLP since the corresponding pK is estimated to be only 6.5. Consequently, E.N-MePLP has difficulty in forming the covalent enzyme-substrate complex with glutamate which in turn leads to a high dissociation constant.

The other reason could be that during the formation of the covalent complex with substrate, the coenzyme ring needs to rotate. This conformational change of the coenzyme ring is evident in the difference map of the AAT-α-methylaspartate complex (Arnone, private communication, 1981; Jansonius, private comm., 1981). In the case of E.N-MePLP, the bulk
of the N-methyl group may simply prevent such movement of the coenzyme ring. Again, the result will be a lack of substrate binding.

**The spectra of enzyme-substrate intermediary complexes**

Spectra have been recorded for native AAT at saturating concentrations of both substrates, glutamate and α-ketoglutarate (Jenkins and D'Ari, 1966b; Fonda and Johnson, 1970). Absorption bands are seen at 490 nm, 430 nm, 368 nm and 330 nm. The apparent molar absorptivities for these bands are approximately 400, 500, 2800 and 6000. If the intrinsic molar absorptivities are taken as 40,000 (Ulevitch and Kallen, 1977), 7000 and 8000 (which are the molar absorptivities of the protonated and deprotonated forms of AAT, Fonda and Johnson, 1970) and 8000, respectively then the relative amounts of the complexes at equilibrium are 0.001, 0.07, 0.3 and 0.75.

A similar spectrum for the glutamate complex of E.N-MePLP was estimated (Figure 40). The apparent molar absorptivities of the bands at 490 nm, 430 nm, 368 nm and 330 nm are about 1000, 4000, 2500 and 2500. With their intrinsic molar absorptivities taken as 40,000, 7200, 9000, and 9000 (the molar absorptivities of the protonated and deprotonated forms of E.N-MePLP being 7200 and 9000), the relative amounts of the intermediates at equilibrium are 0.03, 0.56, 0.27 and 0.27. These figures are of the same order of magnitude as those of the native enzyme. This fact suggests that N-methylation of the coenzyme has altered only slightly the equilibria between the enzyme substrate intermediary complexes. The corollary is that N-methylation affected
equally the forward and reverse rates of the transamination reaction.

**The activity of E.N-MePLP**

With the dissociation constants determined for various ligands, it is possible to estimate the maximal velocity of E.N-MePLP. The data reported in Table 18 were collected with the concentration of aspartate below the substrate dissociation constants. Also, the concentration of \( \alpha \)-ketoglutarate is above the value of the dissociation constant, \( K_4 \), of the unproductive complex with the aldimine form of E.N-MePLP. Assuming that the substrate dissociation constant for aspartate is 0.8, which is one fourth the value of \( K_5 \) for glutamate, the maximal velocity, \( V_{\text{max}} \), of the enzyme can be estimated using the Michaelis-Menten equation:

\[
V_{\text{max}} = \frac{v(K_5(1+[kg]/K_4))}{[aspartate]+1}
\]

where \( v \) is the velocity of E.N-MePLP given in Table 18. Therefore, the calculated value of \( V_{\text{max}} \) is 0.15 mol/s/mg. This compares with the corresponding value of 5.5 mol/s/mg for native AAT (it is necessary to use the value for native AAT determined at 0.02 M aspartate since high concentrations of substrates leads to substrate inhibition). It can be seen that E.N-MePLP has about 3% the activity of the native enzyme. The fact that maximal activity comparable to that of the native enzyme is not obtained for E.N-MePLP fully saturated with substrate means that other factors must also be involved other than the low affinity of the substrate. An obvious one is that steric hindrance introduced by the
size of the N-methyl group hampers conformation flexibility that is needed in certain steps of the reaction. This is not inconsistent with the observed low value of $pK_a$ for an E,N-MePLP dicarboxylate complex already discussed.

The dissociation constant for N-MePLP, $K_{co}$

With the data hitherto reported for E,N-MePLP, it is possible to calculate the dissociation constant for N-MePLP, $K_{co}$, with the aid of Scheme 17:

![Scheme 17](image)

Here, the equilibrium constant for the nonenzymatic transamination, $K_{nt} = [\text{N-MePLP}][kg]/[\text{N-MePLP}][\text{Glu}] = 41$; the equilibrium constant for the enzymatic transamination, $K_e = [E_M][kg]/[E_L][\text{Glu}] = 3.5 \times 10^{-5}$ and the dissociation constant for N-MePMP, $K_g = [E][\text{N-MePMP}]/[E_M] = 5.1 \times 10^{-5}$ M. Then, the dissociation constant for N-MePLP, $K_{co} = [E][\text{N-MePLP}]/[E_L] = K_e K_g/K_{nt} = 4.4 \times 10^{-11}$ M.
It has already been discussed under Literature Review that because of the great tenacity with which an aldehydic coenzyme analog is bound to the apoenzyme of AAT, the binding reaction is virtually irreversible. Therefore, it is exceedingly difficult to estimate an equilibrium constant for the association of the coenzyme analog with the apoprotein (Fonda, 1971). The value of $K_{CO}$ calculated above for N-MePLP is a true equilibrium dissociation constant. It is the first of such a constant reported for any coenzyme analog in the aldehyde form for any vitamin $B_6$ dependent enzyme.

**Reaction of E.N-MePLP with erythro-β-hydroxy-L-aspartate**

The unique characteristics for the reaction of **erythro-β-hydroxy-L-aspartate** with native AAT are well recognized. This pseudosubstrate has very high affinity (below 20 μM) for both the aldimine as well as the amine forms of AAT. It reacts with the aldimine form of AAT to give a mixture of enzyme substrate intermediary complexes, of which the quinonoid intermediate is the predominant species (Jenkins, 1964). It is slowly transaminated by the enzyme with a forward rate being two orders of magnitude slower than that of aspartate. However, it is still not understood why only this amino acid and no other possesses these properties.

As already reported in this thesis, E.N-MePLP reacts with both aspartate and glutamate in similar manners as native AAT. Therefore, it is surprising that combination of E.N-MePLP and **erythro-β-hydroxy-L-aspartate** gives only barely detectable amounts of the quinonoid intermediate. Instead, a new band is observed at 400 nm ($25.0 \times 10^3$ cm$^{-1}$).
Since an absorption band in the range 360 nm to 430 nm is due to a chromophore with an sp$^2$ hybridized 4'-carbon atom and an ionized 3-phenolic oxygen (Metzler et al., 1980), it is tempting to assign the band at 400 nm to a form of bound Schiff base of hydroxyaspartate and N-MePLP. This form of Schiff base may be nonplanar since the band at 400 nm possesses a negative circular dichroism which is in contrast to the lack of any CD in the substrate Schiff base in the native enzyme.

A detailed kinetic study is yet to be performed. However, qualitatively in comparison to the reaction with glutamate, erythro-β-L-aspartate seems to react more slowly with E.N-MePLP. With glutamate at 5 mM, equilibrium is attained in less than two hours, but with 6 mM hydroxyaspartate, the reaction is not at equilibrium even after 12 hours.

The curious thing about the reaction of erythro-β-hydroxy-L-aspartate with E.N-MePLP is that the absorption band at 490 nm appears to remain constant throughout the reaction while both the bands at 376 nm and 400 nm decrease to give rise to the absorption band at 330 nm. No isosbestic point is observed. It seems like that the enzyme combines with the substrate to give a complex absorbing at 400 nm. This then reacts slowly to give a species absorbing at 330 nm via the quinonoid intermediate as is summarized in the following scheme:

$$E.N-MePLP + Substrate \xrightarrow{\text{A}_{400}} A_{490} \xrightarrow{\text{A}_{330}} A_{330}.$$ 

More data are needed to clarify the reaction mechanism. Nevertheless,
it is interesting that while N-methylation of the coenzyme does not affect the enzymatic transamination greatly, it seriously altered the reaction with erythro-β-hydroxy-L-aspartate.

**Reaction of E.O-MePLP with aspartate**

E.O-MePLP has been previously prepared by Furbish et al. (1969) and Mora et al. (1972). They both reported the reaction with enzyme-bound substrates to produce spectra characteristic of the quinonoid intermediate. In this thesis, the formation and breakdown of the quinonoid intermediate were examined. The major finding is that the reaction is pH dependent with an associated pK of 8. This is interesting, since both the maximal velocity as well as the equilibria between the enzyme-substrate intermediary complexes in native AAT lack pH dependence (See Literature Review). However, it must be remembered that the reaction of E.O-MePLP with aspartate proceeds at a rate six orders of magnitude slower than for the native enzyme. Conceivably, certain intermediate steps in the pathway become sensitive to pH when the reaction is drastically retarded. It can also happen that the pK of an ionizable group involved is normally outside the range studied but is shifted to about 8 by virtue of some unusual interaction between O-MePLP and the protein.

The variation of the rate constants with pH for the reaction between E.O-MePLP with aspartate was fitted mathematically to the chemical model of Scheme 13 in which two different pathways, interconvertible by protonation, lead to products. The fact that the observed inverse
relationship between the maximal amount of the quinonoid intermediate formed and the observed rate constant of the reaction necessarily associates the pathway involving the quinonoid intermediate with that having the smaller rate constant.

The existence of the two pathways in the reaction of E.O-MePLP is consistent with certain aspects of the pH-jump experiments reported in Figures 55 and 56. These experiments clearly illustrate that the reaction progresses in spite of the absence of absorbance at 480 nm. This is in agreement with the fact that the observed rate constant for the decay of the quinonoid intermediate is the same as that of the appearance of oxalacetate as assayed by malic dehydrogenase (Figure 53). However, given a faster reaction at low pH, it is hard to reconcile the fact that the curves for the reactions initiated at pH 5 and subsequently jumped to pH 8 are above that for the reaction initiated at pH 8 (Figure 56). In contrast, when the reaction is started at pH 8, then jumped to pH 5 and rejumped once more to pH 8, the curve for the reappearance of the absorbance at 480 nm is below the control experiment performed at a single pH of 8 (Figure 55).

Since two pathways are involved in the reaction of E.O-MePLP with aspartate to form products, the question arises as to whether the quinonoid intermediate is a true intermediate in both pathways. While it is hard to refute that it is in the reaction occurring at low pH, its absence can be explained in terms of a change in the rate determining step in decreasing the pH of the reaction medium. One such possibility is depicted as follows:
Here, Q and Q' represent the colored quinonoid intermediate; A and A' represent two colorless intermediate prior to Q and Q', respectively. Structurally, there is no difference between the primed and the unprimed species except by a single proton. Thus, it can be postulated that deprotonation causes $k_1$ to be greater than $k_2$ resulting in the accumulation of Q. However, protonation of A to A' reverses the relative magnitudes of $k_1$ and $k_2$ such that Q' does not accumulate. The site of protonation is certainly of great interest. There is no compelling evidence requiring protonation to occur at or near the active site. Nevertheless, the $pK_a$ value of 8.1 found for the interconversion between the two pathways in the reaction of E.0-MePLP with aspartate is almost identical to that estimated for the $\varepsilon$-amino group of Lys-258, which is 7.9 (Slebe & Martinez-Carrion, 1976). Furthermore, from X-ray crystallographic studies of the mitochondrial isoenzyme of native AAT, Eichele (1980) has postulated that the phenolate side chain of Tyr-70 from the neighboring subunit is responsible for the abstraction of $\alpha$-H from the substrate Schiff base to generate the quinonoid intermediate.
while subsequent reprotonation at the 4'-position of the coenzyme is performed by the protonated ε-amino group of Lys-258. Therefore, it is tempting to assign the observed pK of 8.1 in the case of E.O-MePLP also to the ε-amino group of Lys-258.

The binding of substrate determined at 5.5 °C is complicated as evident by the nonlinearity in the Scatchard plot (Figure 57b). Nevertheless, the similarity in the shapes between the curves for studies carried out at different pH implies that the equilibrium dissociation constant of substrate is not sensitive to pH. This means that the quinonoid intermediate, which can be reversibly protonated, is probably not in rapid equilibrium with the substrate and the free enzyme. Mechanistically, how the nonlinear behavior observed in substrate binding fits with the formation and turnover of the quinonoid intermediate is not clear at this stage.

Regardless the kinetic mechanism involved, the binding of substrate to E.O-MePLP is weak. The data in Figure 57 show that even at 2 M aspartate, saturation is not achieved. Ulevitch and Kallen (1977) estimated a value of 40,000 for the apparent molar absorptivity of the quinonoid intermediate. With this value, it can be seen that, to a first approximation, the dissociation constant for aspartate is on the order of 1 M.

The explanation advanced earlier in this discussion for reduced affinity of E.N-MePLP for the substrate can also apply to that of E.O-MePLP. Namely, the formation of the first covalent complex is
impeded because proton transfer between the protonated \( \alpha \)-amino group of the substrate and the imino nitrogen of the bound coenzyme cannot take place. This is consistent with the low value of \( pK_a \) for the imino nitrogen of E.0-MePLP as has already been discussed.

Conclusion

The object of this work was to investigate the reasons for the inactivity of aspartate aminotransferase reconstituted with methylated vitamin \( B_6 \) phosphates. It was hoped that the experiments would provide some insight into the interaction between the coenzyme with the protein, and to the possible role of the interacting groups from the protein in catalysis.

For the enzyme reconstituted with N-methylpyridoxal 5'-phosphate, no gross difference from the native enzyme was found in its reaction with anions and inhibitors, but its affinity for substrate was drastically diminished. Parallel studies were made of the properties of the free N-methylated coenzyme and its Schiff base together with those of pyridoxal 5'-phosphate and its Schiff base. It was postulated that due to N-methylation, and the resultant lack of hydrogen bonding between the ring nitrogen of the coenzyme with the carboxyl group from the side chain of Asp-222, a change was induced in the charge distribution within the coenzyme. This in turn led to a poor interaction between the enzyme and the substrate. The substrate dissociation constants for glutamate and \( \alpha \)-ketoglutarate were found to be 3.2 M and \( 1.1 \times 10^{-4} \) M.
Thus, under the assay condition for maximal activity for the native enzyme at 0.0067 M for both aspartate and α-ketoglutarate, there is little formation of enzyme-substrate complex for the reconstituted enzyme. Hence, the activity is immeasurably low. The release of the amine form of the N-methylated coenzyme also contributes to inactivity.

With the estimated dissociation constants for substrates, the hypothetical maximal activity of the enzyme reconstituted with N-methylpyridoxal 5'-phosphate, and saturated with both glutamate and α-ketoglutarate, was calculated to be about 3% that of the native enzyme. However, the equilibria between the enzyme-substrate intermediary complexes of the reconstituted enzyme were not greatly altered. It was concluded that steric hindrance of the bulky N-methyl substituent decelerated the reversible transamination reaction to a similar extent in both directions.

With aspartate aminotransferase reconstituted with N-methylpyridoxal 5'-phosphate, the difference in the mode of binding between a substrate and a dicarboxylate inhibitor is exaggerated. This emphasizes the fact that caution must be exercised when drawing conclusions regarding substrate binding using data from studies with inhibitors.

In this thesis, the study with aspartate aminotransferase reconstituted with O-methylpyridoxal 5'-phosphate was less extensive. Low affinity of the reconstituted enzyme for substrate was also observed as was the release of the amine form of the O-methylated coenzyme. In addition, the turnover of substrate was retarded by at least six orders
of magnitude compare to the native enzyme. However, precisely because of the slow rate of reaction, the quinonoid intermediate was observed transiently. Kinetic analysis showed that the turnover of the quinonoid intermediate in the O-methylated analog reconstituted enzyme was sensitive to pH. An apparent pK of about 8 was found. This is in contrast to the well known pH independence of catalytic transamination by aspartate aminotransferase.

From the studies reported in this thesis, it is evident that the enzyme reconstituted with so-called inactive coenzyme analogs can undergo reversible transamination albeit at much reduced rates. However, this offers an opportunity to study some reactions that are normally too fast to be observed.
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