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Interaction of coenzyme and substrate analogs
with glutamate decarboxylase

by

John Joseph Likos

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INTRODUCTION

Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her working apart from the beaten path.

William Harvey, 1847

The multiplicity of amino acid enzymatic reactions which are dependent upon pyridoxal phosphate as a coenzyme is impressive. Such reactions include transamination, $\alpha$ or $\beta$ decarboxylation, racemization, elimination of the $\alpha$ proton together with either a $\beta$ or $\gamma$ substituent, reactions that are $\alpha,\beta$ eliminations followed by addition to the double bond and the cleavage of $\beta$-hydroxyamino acids to glycine and carbonyl compounds. Most of the reactions catalyzed by $B_6$ enzymes could be demonstrated in nonenzymatic model systems (Metzler et al., 1954) which confirmed the mechanism first postulated by Braunstein and Shemyakin (1953). This general mechanism encompassed all of the known enzyme-catalyzed reactions.

Important differences exist between the model systems and the enzymatic reactions. The enzyme-catalyzed reaction is very specific in terms of substrate used and type of reaction catalyzed. Additionally the enzymatic reactions occur at rates several magnitudes of order greater than their nonenzymatic counterparts. The reaction and substrate specificity and rate enhancement, which are characteristic of all enzymatic reactions, must be due to the protein. How does the protein "tame" the versatility of the coenzyme; what factors make a $B_6$ enzyme or any enzyme an exceptional catalyst? Many methods have been used to perhaps answer these questions.
Information concerning the chemical properties of a residue within a protein may be obtained through the use of active site directed reagents. These 'affinity labels' differ from nonspecific protein modifying reagents in that they possess substrate-like features in addition to a chemically reactive group. Selectivity of modification of the active site is based upon the premise that the affinity label takes advantage of the normal substrate-enzyme interactions to ensure that a high local concentration of reagent is at the active center. Thus the formation of this noncovalent complex is solely dependent upon forces that govern binding. The action of modification by an affinity label depends upon the ability of the reactive group in the label to form a stable covalent linkage to an amino acid residue at the active site. The amino acid so modified may or may not be essential for activity, but it can be assumed with some certainty that it is near the active site.

In addition to substrate analogs that probe the active center of an enzyme by means of irreversible modification much can be learned about the geometry of the active center and residues involved in substrate binding through the use of reversible inhibitors. For example, the binding of glutamate and its analogs to glutamine synthetase has been studied (Meister, 1968; Gass and Meister, 1970) and it appears that glutamate binds to the enzyme in the fully extended conformation.

Tentative answers as to which amino acid residues define the active center of an enzyme, that is those residues involved in substrate binding and catalysis and the spatial organization of these residues with respect to the substrate, can be gleaned from X-ray diffraction. Yet even when
the amino acid residues that constitute the active center have been identified, little is known about their specific chemical properties within the protein. Furthermore crystallographic pictures are static and convey no information concerning dynamic changes which may occur during catalysis. It cannot be assumed that knowledge of the structure of an enzyme is knowledge of its mechanism. However structural information is a prerequisite to mechanism.

Ideally one would like to observe the intermediate species formed upon reaction of the normal substrate with an enzyme. However these reactions by their very nature are quite rapid and any bonds formed between substrate and enzyme are usually labile and transitory, although in certain systems it has been possible to trap these intermediates by stabilizing the enzyme-substrate linkages. An example of this 'substrate labeling' is the demonstration of Schiff base formation upon interaction of dihydroxyacetone phosphate with aldolase. Reduction of the enzyme-substrate intermediate and hydrolysis of the protein yielded one mole of N\textsuperscript{6}-\textbeta -glyceryl-L-lysine per mole of enzyme.

Amino acids that are modified during the course of catalysis of a substrate or substrate analog can give information as to changes in chemical reactivity of these residues in the catalytic intermediates. One such type of modification has been termed syncatalytic modification, that is, synchronous with catalysis of a substrate and in the presence of the appropriate modifying reagent, an amino acid, that is not normally susceptible to modification, becomes reactive and is amenable to modification.
An interesting example of this type of modification has been observed for aspartate aminotransferase. This enzyme has five sulfhydryl groups; two are on the surface of the molecule as evidenced by their susceptibility to modification, two are unreactive, and one is syncatalytically reactive (Birchmeier et al., 1973a, 1973b). With the pyridoxal phosphate form of the enzyme and in the presence of \(\alpha\)-ketoglutaric acid and L-glutamate, one sulfhydryl group is 100 times more reactive than in the absence of substrate. With \(\alpha\)-methylaspartate, which forms the aldime intermediate (Jenkins et al., 1959), or with \(\beta\)-hydroxyaspartate, which forms the semiquinonoid intermediate (Jenkins, 1961), as substrates, the rate of modification is tenfold lower than that when the normal substrates are present. Thus it was concluded that the sulfhydryl group had the highest reactivity at the ketimine stage of catalysis. The sulfhydryl modified has been identified as cysteine 390 (Birchmeier et al., 1973b).

Although the modifying reagents used are not necessarily active site directed reagents, syncatalytic modifications are the result of changes in the chemical reactivity of the residue modified during catalysis. Changes in chemical reactivity of a residue may be the result of a conformational change occurring during reaction of the enzyme with a substrate or may result from a charge redistribution within the active center during catalysis, the effects of which could be mediated either directly or through the protein to the residue modified.

Perhaps the most interesting way to probe an enzyme during the course of catalysis is through the use of substrate analogs that possess latent reactive groups. Unlike affinity labels, these reagents are not reactive
but require chemical activation by the enzyme during the course of catalysis which then leads to irreversible modification. Thus the enzyme by its specific mode of action catalyzes its own inactivation. This class of inhibitors has been designated as $k_{\text{cat}}$ inhibitors (Rando, 1974) since their mechanism of inactivation depends upon catalytic conversion to a reactive intermediate.

Enzymes with a removable prosthetic group as in most enzyme-coenzyme systems offer yet a second method with which to perturb its normal reactivity. In most cases the introduction of a probing molecule within the protein limits what can be learned when the aim is the elucidation of the chemical properties of the active site with a minimum of perturbation of enzyme structure. However cofactor containing enzymes are well suited to this approach since the introduction of a coenzyme analog offers little perturbation to the system. To this end many pyridoxal phosphate analogs have been synthesized and the apoenzyme-analog complexes studied using a wide variety of physical techniques including $^{31}$P, $^{13}$P, and $^{13}$C nmr.

The problem encountered in the study of enzymatic mechanisms is the detection of fleeting events that occur during catalysis. The use of substrate and coenzyme analogs provides a means of stepping off the "beaten path" with the hope of catching a glimpse of the secret of catalysis. The next section will review the reactions of substrate and coenzyme analogs with various $\text{B}_6$ enzymes. Since the interest in this paper is primarily focused on bacterial glutamate decarboxylase, the chemical and physical properties of this enzyme will be reviewed.
Glutamic Acid Decarboxylase (GAD)

Glutamate decarboxylase was first isolated and characterized by Shukuya and Schwert (1960). Strausbauch and Fisher (1970a) concluded on the basis of an investigation of the terminal amino acid residues of the enzyme, the peptide maps of its tryptic hydrolyzate, sedimentation equilibrium in guanidine HCl and acrylamide gel electrophoresis in sodium dodecyl sulfate that the enzyme was a hexamer composed of identical subunits with a molecular weight of 50,000 per subunit. Electron micrographs of GAD suggested that the molecule is a hexamer with dihedral symmetry in which the subunits are accommodated at the vertices of an octahedron (To, 1971). GAD contains one mole of PLP per subunit bound as a Schiff base to the ε amino group of a lysine residue. Although the enzyme has not been totally sequenced, the primary structure of the PLP binding site has been established as Ser-Ile-Ser-Ala-Ser-Gly-His-Lys-Phe (Strausbauch and Fisher, 1970b).

The spectral properties of GAD as a function of pH have been studied (Shukuya and Schwert, 1960). The absorption spectrum of the active enzyme is characterized by an absorption maximum of 420 nm (23.8 kK) at pH 4.6. This absorption band represents the protonated aldimine existing principally as the ketoenamine rather than the tautomeric phenol (Heinert and Martell, 1963a, 1963b). Above pH 6 the enzyme absorbs at 340 nm (29.4 kK) and is catalytically inactive. The midpoint of the transition is at pH 5.61. The steepness of the titration curve suggested that a simultaneous dissociation of at least four protons occurs.
The structure of the high pH form of the enzyme is uncertain. However, the absorption at 340 nm is thought to represent a geminally substituted compound formed by the addition of some group X to the 4' carbon of the low pH form (Anderson and Chang, 1965; O'Leary, 1971; O'Leary and Brummund, 1974) rather than a Schiff base in an hydrophobic environment which would also absorb in this region of the visible spectrum (Johnson et al., 1970; Heinert and Martell, 1963a, 1963b). Several lines of evidence support this idea. It has been shown (Anderson and Chang, 1965) that the high pH form of the enzyme is resistant to reduction with NaBH₄ and that PLP is released from the protein upon a further increase in pH whereas the low pH form of the enzyme exhibits a spectral shift from 420 nm to 330 nm (30.3 kK) upon treatment with borohydride. Pyridoxyllysine was identified in hydrolyzates of the enzyme reduced at pH 4.8 but not at pH 7.2.

As expected for a protonated imine (Matsushima and Martell, 1967; Shatiel and Cortijo, 1970; Arrio-Dupont, 1970), the low pH form of GAD fluoresces maximally at 490 nm (20.4 kK) when excited at 420 nm (23.8 kK), a Stokes shift of 3400 cm⁻¹ (5). The high pH form of GAD has a maximum fluorescence at 380 nm (26.3 kK) when excited at 340 nm (29.4 kK) or a Stokes shift of 3100 cm⁻¹. Dipolar ionic species which have a saturated 4' carbon show absorption around 330 nm (30.3 kK) and have Stokes shifts of 5000–6000 cm⁻¹ (Shatiel and Cortijo, 1970; Bridges et al., 1966), whereas the neutral tautomer of the protonated imine would be expected on the basis of model studies (Weller, 1955, 1956) to exhibit a Stokes shift of 10,000–11,000 cm⁻¹. The large shift is interpreted as being the result.
of proton transfer from the 3 hydroxyl group to the imine nitrogen in the excited state.

Although the identity of group X remains a subject of speculation, it has been argued that X may be the sulfhydryl of a cysteine residue (O'Leary and Brummund, 1974). Such additions are known to occur in model systems (Heyl et al., 1948; Buell and Hansen, 1960). Titration of native GAD with Ellman's reagent indicated that one out of the ten sulfhydryl groups per subunit was accessible to the solvent (Strausbauch and Fisher, 1970a). This is true for both the high and low pH forms of the enzyme. If X is a sulfhydryl, it must be buried in the protein.

In addition to different covalent structures of the bound coenzyme, it has been concluded from the kinetics of pH jump studies that the low and high pH forms of GAD differ in protein conformation (O'Leary and Brummund, 1974).

One final piece of spectral data concerning GAD deals with the optical activity of the Schiff base absorption band. Although PLP itself is optically inactive, in association with apoGAD there is an induced positive circular dichroism (CD) at 420 nm at pH 4.6 whereas the high pH form shows very little CD (Huntley and Metzler, 1967; Sukhareva and Torchinskii, 1966). This may reflect differences in protein conformation for the two forms. Of interest is that the NaBH₄ reduced enzyme has positive CD at both pH 4.6 and 6.3 which disappears upon denaturation with 8 M urea.

Mechanism of PLP-Dependent α-Decarboxylases

Using bacterial decarboxylases, it was established that the replacement of the carboxyl group was stereospecific. Decarboxylation of the
substrate in $D_2O$ led to the incorporation of one atom of deuterium at the 
$\alpha$ carbon of the product (Mandeles et al., 1954). The monodeuteroamine 
produced is one of a pair of enantiomers, the opposite enantiomer can be 
obtained by decarboxylation of an $\alpha$-deutero amino acid in $H_2O$ (Mandeles 
and Hanke, 1953). These results indicate as well that the $\alpha$ proton of the 
substrate does not exchange. The mechanism of decarboxylation as proposed 
by Metzler et al. (1954) is shown in Figure 1.

The decarboxylation and subsequent protonation has been determined, 
至少在 tyrosine decarboxylase 的情况下，已经确定了这种反应以保持构型的保留，
the added pro-$R$ proton having the same configuration as 
the carboxyl group it replaced (Belleau and Burba, 1960).

It has been argued that in general for the PLP requiring enzymes, the 
$C_{\alpha}$ bond to be broken should be perpendicular to the plane of the cofactor 
imine pi system (Dunathan, 1966). This configuration allows maximum 
sigma-pi overlap of the bond being broken with the pi system of the imine 
ring. The specificity of the various PLP enzymes then is determined by 
the manner in which the protein aligns the amino acid with respect to the 
cofactor.

Reactions of Substrate Analogs with GAD

Under the appropriate conditions the following spectral changes can 
be observed upon reaction of L-glutamate with GAD: 420 nm, +CD (PLP-
protein aldimine); 340 nm, short lived (geminal diamine); 420 nm, -CD 
(PLP-substrate aldimine); 420 nm, +CD (PLP-protein aldimine) (Sukhareva 
and Braunstein, 1971). The addition of glutamate causes, as well, a 
decrease in the fluorescent intensity at 490 nm (420 nm excitation) and
Figure 1. Mechanism of decarboxylation
an increase in fluorescence at 380 nm (335 nm excitation) (Shukuya and Schwert, 1960). These changes are reversible upon depletion of the substrate. What is the structure of the species responsible for the 340 nm absorption and 380 nm fluorescence? As indicated above these spectral properties are consistent with a dipolar ionic species with a saturated 4' carbon.

A short lived 340 nm species is also observed during the reaction of GAD with α-methylglutamate (Sukhareva and Braunstein, 1971; Huntley and Metzler, 1968). The following spectral changes are observed immediately after adding the analog: the 420 nm absorption of the native enzyme decreases and an absorption band at 340 nm appears which is converted back to one at 420 nm. This 420 nm peak is then slowly converted to a peak at 325 nm. This final form of the enzyme was found to be inactive but activity could be restored upon addition of PLP. The species absorbing at 325 nm was identified as PMP. In addition to this decarboxylation-dependent transamination, a normal decarboxylation was also occurring at a rate 100 times faster than that of the transamination.

Both of these reactions require a common carbanion intermediate as shown in Figure 2. Protonation of the α carbon of I leads to the normal decarboxylation products, whereas protonation of the 4' carbon would give PMP, apoenzyme and a carbonyl compound.

Using tetranitromethane as a trapping reagent, evidence has been obtained that carbanion formation does indeed occur during the reaction with α-methylglutamate (Mekhanik and Torchinskii, 1972). However nitroformate formation was not observed during the decarboxylation of glutamate possibly
Figure 2. Proposed carbanion intermediate formed during the reaction of GAD with α-methylglutamate
due to the shorter lifetime of the semiquinonoid intermediate. Thus in addition to causing the proton to be occasionally put on the wrong carbon, the proposed distortion of the active site by the α methyl group (Huntley and Metzler, 1968) may also lead to the stabilization of the semiquinoid intermediate.

The intermediate absorbing at 340 nm is probably the covalent adduct of the Schiff base of the enzyme aldimine and the 420 nm intermediate which follows is the Schiff base of the substrate and PLP. A study of the kinetics of formation and breakdown of these intermediates has indicated that they cannot be part of the reaction sequence for the normal decarboxylation seen during the reaction with α-methylglutamate but are intrinsic to the decarboxylation-dependent transamination (Kao and Metzler, unpublished observations). It is thus possible that these same intermediates observed during the decarboxylation of glutamate are not part of the normal reaction sequence especially in view of the fact that reaction with large amounts of glutamate leads to formation of PMP (Sukhareva and Braunstein, 1971).

Tentative ideas regarding conformational changes that may occur in the substrate during catalysis have been formed through a study of the interaction of the cyclic glutamate analogs with GAD (Sastchenko et al., 1971).

Interaction of the enzyme with III (Figure 3) led to a small decrease in the absorbance at 420 nm and a small increase in absorbance at 340 nm. Following this initial rapid change the 420 nm absorbance disappeared and a new absorbance band appeared at 380 nm which was nondichroic.
Figure 3. *erythro*-γ-cycloglutamate

After 2 hours the spectral changes were almost complete, however the enzyme could be reactivated by glutamate to 80% of its original activity. After 8 hours very little activity could be regained. Thus the latter stages of the inhibition were irreversible.

It was postulated that the 380 nm band observed during the early stages of inhibition by III represented a Schiff base but that the imine double bond was not coplanar with the pyridine ring perhaps due to the fixed conformation of the analog. This was consistent with the isolation of N-pyridoxyl-III after reduction at the early stage of inhibition. From the fact that no decarboxylation of the analog was observed and that only undecarboxylated pyridoxyl-III was obtained upon reduction of the EI complex it was postulated that a change in conformation of the protein, or the substrate or both was required for decarboxylation.

Since the analog had a high affinity for the enzyme it was suggested that the complex formed between the analog and GAD represented the Michaelis complex and the tetrahedral adduct (340 nm form). It should be
noted that in this fixed conformation the carbon chain of the analog is almost fully extended.

Saturation of GAD with glutamate and reduction of the ES complex led to formation of N-pyridoxylpyroglutamate. These results suggest that in formation of the Schiff base between coenzyme and glutamate the γ carboxyl group must be close to the imino nitrogen.

The significance of the dephosphorylation of the coenzyme in these reactions is not clear, but labilization of the phosphoester bond does raise questions concerning the role of the phosphate group in catalysis by PLP requiring enzymes.

Thus it was concluded that the substrate binds in a fully extended conformation and is fully extended in the 340 nm form of the enzyme. If it is assumed that the γ carboxyl is held at a specific binding site, then rotation about the C₆-C₄ bond during elimination of the ε amino group of the lysine would lead to a folding of the substrate which would move the imino nitrogen closer to the γ carboxyl and at the same time place the α carboxyl normal to the pyridine ring as required for decarboxylation.

Both the threeo and erythro isomers of β-hydroxy-L-glutamate are decarboxylated by GAD (Kaneko and Yoshida, 1962). The erythro isomer is attacked more slowly. These results may be understood in light of the work described above in that the hydroxyl group of the erythro isomer would be hindered in rotation by the α hydrogen and one of the γ hydrogens of the substrate during the folding of the substrate.

Although threeo-β-chloroglutamate is not decarboxylated by GAD (Manning et al., 1968), it is a competitive inhibitor whereas the erythro
isomer does not inhibit the enzyme at all (Fonda, 1976).

The interaction of aliphatic mono and dicarboxylic acids with GAD has been studied (Fonda, 1972a, 1972b) in an attempt to determine the structural geometry of the active site. It was found that inhibition by both types of acids was competitive. The dicarboxylic acids were the better inhibitors. Of the dicarboxylic acids studied, glutaric acid was the best inhibitor ($K_I = 0.58$ mM). Of the monocarboxylic acids studied, n-valeric acid was the best inhibitor ($K_I = 3.4$ mM).

The inhibition constants were found to be a function of carbon chain length. In general the substituted dicarboxylic acids were less effective inhibitors than the unsubstituted acids. Interestingly L-aspartate and D,L-α-amino adipic acid did not inhibit the enzyme whereas succinic and adipic acids were effective inhibitors ($K_I = 5.9$ and $1.14$ mM respectively).

The inhibition constant obtained for glutaric acid was found to be a function of pH. Although analysis of these data based solely on the pK values of the acid may be an oversimplification, it appeared that the dianion of glutarate did not inhibit the enzyme. In contrast, evaluation of the $K_M$ and $V_{max}$ for glutamate as a function of pH indicated that glutamate may bind as the dianion. It was concluded from these studies that two free carboxyl groups are needed for effective interaction with the protein and postulated that glutamate binds to the enzyme in a fully extended conformation and that perhaps in the process of binding and/or in the formation of the Schiff base the proton on the amino group of the substrate is transferred to the $\gamma$ carboxyl group, thus forming the monoanion.
Both γ-methylene glutamic acid (Homola and Dekker, 1967) and glutamate-γ-methyl ester (Lupo and Halpern, 1970), both of which can exist as the monoanion only, are substrates for GAD.

Various halocarboxylic acids inhibit GAD (Fonda, 1976). Of interest is that while both 3-bromopropionate and bromopyruvate competitively inhibit ($K_I = 3.5$ and 25 mM respectively) the enzyme, they do not lead to irreversible inactivation, whereas these compounds inactivated aspartate aminotransferase (AAT).

As inactivation of transaminase by bromopropionate progressed the absorption band at 355 nm of the native enzyme decreased and a new peak appeared at 395 nm (Okamoto and Morino, 1972; Morino and Okamoto, 1972a). The inactivation was also accompanied by a loss of the positive CD at 355 nm and appearance of negative CD at 410 nm. The peak at 395 nm was not affected by NaBH₄ nor by L-cysteine sulfinic which normally converts the PLP form of the enzyme to the PMP form. Hydrolysis of the protein yielded Nε-(2-carboxylethyl)lysine as the major product.

Addition of bromopyruvate to the PLP form of AAT does not inactivate the enzyme except in the presence of L-cysteine sulfinic acid or L-aspartate (Okamoto and Morino, 1973). This substrate analog can also act as a keto substrate and thus undergo elimination of bromide anion. The reactions seen are

\[
\begin{align*}
E_{PLP} + L\text{-aspartate} & \rightarrow E_{PMP} + \text{oxalacetate} \\
E_{PMP} + \text{bromopyruvate} & \rightarrow E_{PLP} + \text{pyruvate} + Br^- + NH_4^+.
\end{align*}
\]
Inactivation of the enzyme occurs during reaction 2. During the inactivation the 362 nm (27.6 kK) band of the native enzyme shifts to 330 nm (30.3 kK). The spectrum of the inactivated species did not change upon addition of L-aspartate, L-glutamate, α-ketoglutarate, maleate or succinate. After treatment of the inactivated species with KBH₄ and dialysis against 5 M guanidine-HCl, it was found that most of the coenzyme had been lost although a weak band was present at 325 nm (30.8 kK). This was taken to indicate that the inactivated species existed partly as the PMP form of the enzyme and partly as the ketimine. The three carbon moiety of the bromopyruvate was found covalently bound to Cys³⁹⁰. This cysteine residue is the same one that is syncatalytically modified with sulphydryl reagents and evidence has been presented that the modification of Cys³⁹⁰ by bromopyruvate may also be syncatalytic (Birchmeier and Christen, 1974).

Although bromopyruvate did not alkylate holoGAD, it was effective at inactivating the apoenzyme (Fonda, 1976). Both PLP and phosphate protected the apoenzyme against inactivation. Since phosphate, bromopyruvate, pyruvate and other monocarboxylic acids inhibit PLP binding (Fonda, 1975), it appeared reasonable that bromopyruvate was binding at the site normally occupied by the phosphate group of PLP. The inactivation of the apoenzyme was due to alkylation of a cysteine residue. Reduction and hydrolysis of the modified protein yielded 1-carboxyl-D,L-hydroxy-ethyl-cysteine. Since carboxylic acids substituted on the β carbon (3-bromopropionate, 3-bromo-butyrate and 3-bromopyruvate) led to greater extents of inactivation, it was concluded that the sulphydryl group would have to be within 4 Å of the carboxylate binding site. If the carboxylate and phosphate binding site
are indeed the same, this, then, is perhaps the strongest evidence that the "X" group involved in aldamine formation at high pH may be a sulfhydryl which would be situated near the 4' carbon of PLP although the possibility of differing protein conformations for the apo, holo and high pH forms of the enzyme must be kept in mind.

Although the reactions of β-chloroalanine and L-serine-O-sulfate with GAD have not previously been studied, it is of interest to look at these reactions with AAT.

AAT catalyzes the β elimination of β-chloroalanine with concomitant inactivation of the enzyme (Morino and Okamoto, 1972b; Morino et al., 1974). The elimination products being pyruvate, chloride anion and NH₄⁺. The PMP form of the enzyme and reduced AAT do not react with β-chloroalanine. The rate of inactivation as well as the rate of elimination is accelerated by the presence of formate. The spectral changes that are observed upon inactivation in the absence of formate are that the 362 nm (27.6 kK) band of the native enzyme disappears while the inactivated species gives rise to absorption bands at 333 nm (30.0 kK), 375 nm (26.7 kK) and 420 nm (23.8 kK). Formate has the effect of shifting the 362 nm band of the native enzyme to 345 nm (29.0 kK) at pH 7.8. Additionally there is a small peak at 430 nm (23.2 kK). The pK of the spectral transition between the acidic and basic forms of the enzyme is raised to 7.3 in the presence of formate anion. The spectral changes that are observed in the presence of formate are that the 345 nm band diminishes during the inactivation with the immediate appearance of a band at 333 nm. This initial change is followed by a slow transition to 455 nm (22.0 kK) over
a period of several days. Removal of formate from the reaction mixture yielded the same spectrum as observed for the reaction run in the absence of formate. Reduction of the 333 nm species obtained immediately after addition of substrate and the presence of formate resulted in 90% of the labeled substrate and coenzyme being covalently bound to the protein. Although the 455 nm species was stable toward dialysis (its reduction product absorbing at 365 nm), its linkage to the protein was decomposed during denaturation and reduction. After reduction and hydrolysis of the inactivated enzyme, the amino acid residue modified has been identified as the lysine that normally binds the PLP at the active site (Morino and Okamoto, 1973).

It is of interest to note that the inactivation of AAT by bromopyruvate results in the formation of a covalent bond between a sulfhydryl group and the β carbon of the label bound to the PMP form of the enzyme, whereas the inactivation mediated by β-chloroalanine results in the formation of a covalent bond between a lysyl residue and the β carbon of the label bound to the PLP form of the enzyme. Also of interest is that the reaction of β-chloroglutamate with AAT does not result in the inactivation of the enzyme, although the enzyme is catalyzing the elimination of the substrate, the products being α-ketoglutarate, Cl⁻, and NH₄⁺ (Manning et al., 1968).

L-serine-Ο-sulfate undergoes β elimination in the presence of AAT (John and Fasella, 1969). The enzyme is inactivated during the reaction. The products of the elimination reaction are pyruvate, SO₄²⁻, and NH₄⁺ formed in equimolar amounts. In addition the enzyme slowly transaminates
the substrate analog yielding pyruvate sulfate and the PMP form of the enzyme. The presence of other α keto acids increases the rate of β elimination and inactivation by transaminating with the PMP form of the enzyme thus regenerating the PLP form. Although the spectral changes occurring during the reaction have not been presented, the inactivated species does have an absorption maximum at 345 nm (29.0 kK) at pH 8.0. The presence of thiosulfate in the reaction mixture protects the enzyme against inactivation, presumably by β addition of thiosulfate to the aminoacrylate intermediate produced during the reaction (Cavallini et al., 1973). L-cysteine-S-sulfonate has been identified as the product of this reaction. During the reaction labeled substrate becomes bound to the lysine residue at the active site and to Cys^390 (Bossa et al., 1973). The structure of this chromophore is not known.

These reactions are interesting. Why should a transaminase catalyze a β elimination. One point to remember is that in order to facilitate elimination of the β substituent there must be maximum overlap of the sigma bond to be broken and the pi system of the ketimine-pyridine ring. This requires that the β substituent be perpendicular to the pyridine ring. This conformation would minimize orbital motion in reaching the transition state. Perhaps these analogs bind to the protein in this conformation or it is obtained during catalysis. Since labilization of the α proton to form a ketimine is common to both the transaminases and those enzymes that catalyze β eliminations, it may not be all that surprising that a transaminase would catalyze an elimination provided the substrate had a suitable leaving group on the β carbon.
Mechanism of Binding of PLP to Apodecarboxylase and Reconstitution with PLP Analogs

The chemical versatility of PLP in model nonenzymatic systems is impressive. Yet perhaps even more impressive is the way the apoprotein can govern this versatility. For example depending upon the apoenzyme, the Schiff base between PLP and aspartate can undergo transamination, loss of the α carboxyl group or loss of the β carboxyl group. In addition to influencing reaction specificity, the apoprotein greatly increases the rate of reaction and imposes substrate and stereospecificity on the reaction. In order to understand these effects of the apoenzyme, knowledge of the interactions between apoenzyme and coenzyme is necessary. One general approach taken, in particular to determine the importance of various functional groups of the coenzyme for binding and activation of the apoprotein, is the study of the interactions of the apoenzyme with pyridoxal analogs.

Mechanism of PLP binding

From a kinetic study of the reconstitution of apoGAD with PLP it was concluded that the binding of coenzyme was a two step process (O'Leary and Malik, 1972).

\[
\begin{array}{c}
\text{ApoGAD} + \text{PLP} \rightleftharpoons [X] \rightarrow \text{Holoenzyme}
\end{array}
\]

The binding of 4-deoxypyridoxine phosphate was also a two step process. Since this analog cannot form a Schiff base the kinetic mechanism had to be expanded.

\[
\begin{array}{c}
\text{ApoGAD} + \text{PLP} \rightleftharpoons [X]_1 \rightleftharpoons [X]_2 \rightarrow \text{Holoenzyme}
\end{array}
\]
The kinetic constants for binding of both analogs were similar. This would not be expected if Schiff base formation was the rate determining step. It was concluded that the rate determining step, $k_2$, was a protein conformational change. This conformational change may explain the slower rate of Schiff base formation for the protein when compared to nonenzymatic model systems.

**Binding of PLP analogs to apoGAD**

As the holoenzyme is specific in its requirements for a substrate, the apoenzyme is specific with regard to cofactor. In general, of the PLP analogs studied, very few led to reactivation of the enzyme. However most appeared to bind as evidenced by spectral changes or inhibition of reconstitution with PLP. Consideration of the physical properties of the apoenzyme-analog complexes along with permissible conformations of a series of related analogs may allow tentative conclusions concerning the orientation of the various cofactor functional groups within the active site. Lack of activity may reflect differences in the electronic properties or the inability of the analog to undergo necessary conformational changes during the catalytic event.

**Analogs modified at the 2 position**

One of the analogs that gives appreciable reactivation (20%) is 2-norpyridoxal phosphate (Mekhanik et al., 1972). The complex possessed $+\text{CD}$ at 420 nm, pH 4.6 and at pH 6.7 a small $+\text{CD}$ band was seen at 340 nm. The absence of the 2-CH$_3$ group did not alter the $K_M$ for glutamate, however the $V_{\text{max}}$ was fivefold lower. Thus this complex is similar to the
holoenzyme. Since the 2-CH₃ does not participate directly in the chemical transformation of the substrate it may be that it is important in providing an anchor point for conformational rearrangements in the active site during decarboxylation. Other analogs, 2',2'-dimethyl, 2'-n-propyl and 2'-isopropyl pyridoxal phosphate did not reactivate the enzyme.

**Analogs modified at the 3 position**

3-O-Methyl and 3-deoxy pyridoxal phosphate do not form a Schiff base with the apoprotein and both complexes were found to be devoid of activity (Mekhanik et al., 1972; Fonda, 1971; O'Leary and Malik, 1972). The 3-OH group is essential for Schiff base formation and catalytic activity.

**Analogs modified at the 4 position**

As expected these analogs do not lead to reactivation of the apoenzyme due to the absolute requirement of the 4 formyl group. However these analogs are relatively good inhibitors of reconstitution with PLP, indicating that they do interact with the protein suggesting that factors other than covalent interactions are important in binding.

**Analogs modified at the 5 position**

These analogs may enable delineation of the role of the phosphate in catalysis and perhaps give an indication of the orientation of the phosphate group with respect to the pyridine ring of PLP.

5'-Methylpyridoxal phosphate was found to reactivate the apoprotein to the extent of 20% (Mekhanik et al., 1972). The apo-analog complex absorbed at 412 nm (Fonda, 1971) with +CD at pH 4.6. The \( K_M \) for glutamate is slightly higher than that of the holoenzyme while the \( V_{max} \) is four
times lower. Thus this complex like that of 2-norpyridoxal phosphate is similar in a number of properties to the native enzyme but differs by a substantially lower catalytic effectiveness. The methyl group introduces a restriction in the movement of the phosphate group which may account for the lowered $V_{\text{max}}$.

5'-β-(5'-Deoxypyridoxal)-ethyl phosphate binds to the apoenzyme. The complex absorbs at 417 nm and is devoid of catalytic activity (Fonda, 1971; Mekhanik et al., 1972). 5-Nor-5-methyleneypyridoxal phosphate inhibits the binding of PLP and the complex is inactive (Fonda, 1971). It is evident that the distance and/or orientation of the phosphate is important in restoring activity.

Both 5-nor-5-β-carboxyethylpyridoxal and 5-nor-5-β-carboxyvinylpyridoxal bind to the apoprotein (Mekhanik et al., 1972). The saturated analog possesses +CD at 420 nm while the unsaturated analog exhibited a +CD band at 435 nm. Neither complex is active.

**Analogs modified at the 6 position**

Both 6-methylpyridoxal phosphate and 2-nor-6-methylpyridoxal phosphate inhibit the reconstitution of the apoenzyme (Mekhanik et al., 1972). 2-Nor-6-methylpyridoxal phosphate was shown to form a Schiff base and possessed a +CD band at 434 nm. Both analogs led to 4-5% reactivation. The low catalytic activity may be due to the fact that the 6-CH$_3$ group limits the conformations that the phosphate may have due to steric interactions.

In general the use of coenzyme analogs has indicated that PLP interacts with the apoprotein through all of its functional groups. This
suggests that PLP binds to the protein with only one conformation. Knowledge of this conformation is prerequisite for understanding the mechanism of PLP enzymes.

In an effort to determine this conformation the conformation of the PLP oxime imino group in solution was determined by nmr by making use of the nuclear Overhauser effect. Using the experimentally determined value of $\chi$ (Figure 4), the favorable conformations of PLP imine and its analogs were theoretically calculated (Tumanyan et al., 1974).

For 61% of the total conformers $\chi = 0^\circ \pm 20^\circ$. When $\chi = 0^\circ$ the imino bond is in conjugation with the ring and can hydrogen bond with the phenolate anion. This also permits full variation of values for $\phi$ and $\psi$. The conformation calculations were done for $\chi = 0^\circ$ and $180^\circ$. The results are shown in Table 1 for $\chi = 0^\circ$. The conformational maps that were obtained show seven favorable conformations that form three distinct groups with each group separated by an energy barrier of approximately 15 kcal/mole. Group 1 contains conformations I, IIIa, and IIIb; Group 2, conformations IIa and IVa; and Group 3, conformations IIb and IVb. The separation of the seven conformations into these groups is due to the high energy barrier for rotation about the angle $\phi$ (15 kcal/mole), whereas rotations about $\psi$ have a much lower energy barrier (3.5-5 kcal/mole). A similar map was obtained for $\chi = 180^\circ$, the only difference being in the depth of the minima.

Calculations for 6-methylpyridoxal phosphate indicate that the presence of the methyl group prohibits conformations with $\phi = 0^\circ$, that is conformations I, IIIa, and IIIb. For the R isomer of 5'-methylpyridoxal
Figure 4. Conformation of pyridoxal aldimine with $\chi = 0^\circ$, $\psi = 0^\circ$ and $\phi = 0^\circ$

Table 1. Permitted conformations of pyridoxal oxime at $\chi = 0^\circ^a$

<table>
<thead>
<tr>
<th>Conformation</th>
<th>$\phi$</th>
<th>$\psi$</th>
<th>Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$0^\circ$</td>
<td>$180^\circ$</td>
<td>$-3.61$ kcal/mole</td>
</tr>
<tr>
<td>IIa</td>
<td>$120^\circ$</td>
<td>$180^\circ$</td>
<td>$-4.38$</td>
</tr>
<tr>
<td>IIb</td>
<td>$240^\circ$</td>
<td>$180^\circ$</td>
<td>$-4.38$</td>
</tr>
<tr>
<td>IIIa</td>
<td>$20^\circ$</td>
<td>$80^\circ$</td>
<td>$-3.44$</td>
</tr>
<tr>
<td>IIIb</td>
<td>$340^\circ$</td>
<td>$280^\circ$</td>
<td>$-3.44$</td>
</tr>
<tr>
<td>IVa</td>
<td>$120^\circ$</td>
<td>$300^\circ$</td>
<td>$-5.18$</td>
</tr>
<tr>
<td>IVb</td>
<td>$240^\circ$</td>
<td>$60^\circ$</td>
<td>$-5.18$</td>
</tr>
</tbody>
</table>

$^a$From Tumanyan et al. (1974).
phosphate conformations I, IIb, IIIa and IVb are retained while for the S
isomer conformations I, IIa, IIIb and IVa are possible.

Interaction of apotransaminase with these conformationally restricted
analogs was then studied. The idea being that if the analog interacted
with the apoprotein and could show some ability to reactivate the enzyme,
then the analog had probably assumed the conformation of the PLF coenzyme.
The conformations prohibited in the analog could then be excluded from
those allowed for PLP when bound to the active site.

Interaction of apotransaminase with 6-methyl and with a racemic mix­
ture of 5'-methylpyridoxal phosphate led to Schiff base formation with
50-60% reactivation. It was determined that only one isomer of the 5'-
methylpyridoxal phosphate bound to the protein. 5-Nor-5-β-carboxyvinyl-
pyridoxal phosphate for which ψ is fixed at 180° formed a catalytically
inert complex. However it was bound to the apoenzyme as a Schiff base and
could interact with glutamate. Based on these results it was concluded
that φ was equal to 120° or 240° depending upon the absolute stereochemis­
try of the isomer of 5'-methylpyridoxal phosphate which had reactivated
the enzyme, that ψ = 180°.

Perhaps an additional piece of evidence for the value of φ comes from
the reaction of pyridoxal sulfate with apotransaminase. Pyridoxal sulfate
covalently labels the apoprotein through a mechanism involving elimination
of sulfate (Yang et al., 1974). For elimination of the 5' sulfate to
occur readily, φ should be 90° or 270° which closely agrees with the pro­
posed values of 120° or 240°. It was estimated that a change in φ by 30°
would require 1.5-2 kcal/mole (Tumanyan et al., 1974).
Although it was concluded that the carboxyvinyl analog bound to the protein in the same manner as did PLP, it has been demonstrated that interaction of this analog with apotransaminase leads to irreversible modification of the protein (Miura and Metzler, 1976). The mechanism shown in Figure 5 is similar to that proposed for the inactivation of apotransaminase by pyridoxal sulfate. This mechanism requires that $\phi$ be $0^\circ$ or $180^\circ$. The initial complex of apoenzyme with the carboxyvinyl analog absorbs at lower energy (385 nm) than does the saturated carboxyethyl analog (367 nm) (Furbish et al., 1969), indicating, perhaps, conjugation of the 5' unsaturated site with the ring which would require $\phi = 0^\circ$ or $180^\circ$.

Of interest is that complexes of apotransaminase with either 5-nor-5-\(\beta\)-vinylpyridoxal phosphonate or 5-nor-5-\(\beta\)-ethylpyridoxal phosphonate absorb at the same energy for the pyridoxal form of the enzyme indicating a lack of conjugation of the unsaturated site with the ring, however for the pyridoxamine form of the enzyme the unsaturated analog absorbs at lower energy than does the saturated analog (Metzler and Miura, unpublished observations). It appears that the conformation of the 5 substituent is different for the two forms of the enzyme indicating perhaps a rotation about $\phi$ during catalysis.
Figure 5. Inactivation of aspartate aminotransferase by 5-nor-5-β-carboxyvinylpyridoxal phosphate (Miura and Metzler, 1976)
EXPERIMENTAL

Enzyme

L-glutamic acid decarboxylase (GAD), E.C. 4.1.1.15, was isolated from
E. coli (ATCC 11246) as previously described by Witte (1971) and as modi­
fied by Fonda and DeGrella (1974). The bacteria were grown using the
culture medium described by Huntley and Metzler (1967).

ApoGAD was prepared according to the procedure of Fonda and DeGrella
(1974). The holoenzyme in 0.2 M pyridine HCl, pH 4.6 was resolved by the
addition of solid α-methylglutamate to a concentration of 0.1 M. The pH
was maintained at 4.6 by addition of 1 M potassium phosphate, pH 7. The
solution was gently stirred at room temperature for 1 hour. Any precipi­
tation was removed by centrifugation. The apoenzyme was then dialyzed
overnight against 0.1 M piperazine HCl, pH 4.6 containing 10% (v/v)
glycerol and 1 x 10^{-4} M dithiothreitol. Apoenzyme prepared in this manner
showed less than 1% of its original activity and could be reactivated to
80-90% of its original activity with PLP. All experiments with the apo­
enzyme were done in the piperazine HCl buffer. The addition of glycerol
stabilized the apoGAD (Sukhareva and Tichonenko, 1972).

For the analog binding experiments the amount of analog added was
calculated such that the molar ratio of analog to apoenzyme was equal or
less than the amount of active apoenzyme. The reconstitution with analog
was done at 10°C.

Molar concentrations of the holo and apo forms of the enzyme were
determined from the absorbance at 23.8 kK (10,000 M^{-1}cm^{-1}) and at 35.5 kK
(86,100 M^{-1}cm^{-1}) (Fonda, 1971).
Decarboxylase activity was assayed using a differential respirometer to follow CO$_2$ production. Assays were conducted at 25°C in 0.1 M pyridine HCl, pH 4.6 containing 20 mM L-glutamate. Three ml of the assay mixture were placed in the main compartment of the respirometer flask and the enzyme in the side arm. After equilibration at 25°C for 5 minutes the contents were mixed and the CO$_2$ evolved was measured at 1 minute intervals. The amount of enzyme used was such that the rate of CO$_2$ production did not exceed 10 µl/min. Under these conditions the rate was linear for 8-10 minutes.

Spectra

Absorption spectra were measured with a Cary 1501 recording spectrophotometer interfaced through a Cary-Datex digital output to an IBM card punch. Spectra were corrected for baseline errors and small amounts of turbidity. The data were replotted automatically as molar absorptivity or absorbance vs. wavenumber.

Circular dichroism (CD) spectra were measured with a JASCO Model ORD/UV-5 spectrophotometer equipped with a Sproul Scientific SS-20-2 modification. The ellipticity recorded by the instrument was manually transcribed at 2 nm intervals onto IBM cards and the spectra replotted by the computer as molar ellipticity vs. wavelength.

Nuclear magnetic resonance (nmr) spectra were obtained using a Varian A-60, Hitachi-Perkin Elmer R20B, or the Bruker 90 operating in the fourier transform mode. Mass spectra were obtained using an AEI MS-902 mass spectrometer.
Kinetics

Rate constants were calculated by plotting the log of the difference of the concentration related variable (absorbance) and the value after the reaction is complete (7-8 half lives) versus time. The observed rate constant was determined from the slope of the least squares line fitted to the data.

For the biphasic reactions the least squares line fitted to the slower reaction was subtracted from the observed data for early times of reaction and the data points thus obtained for the faster reaction were then fitted with a least squares line and the $k_{obs}$ determined from the slope.

Values of $k$ and $K$ in the double reciprocal plots were calculated from the Y intercept and the slope obtained from a least squares fit of the reciprocal of the $k_{obs}$ vs. the reciprocal of the substrate concentration.

Isolation and Purification of L

Typically to 30 ml of 1.2 x $10^{-3}$ M GAD (180 mg) in 0.1 M pyridine HCl, pH 4.6 was added 80 mg of L-serine-O-sulfate (1.2 x $10^{-2}$ M). The reaction mixture was incubated at 4°C until the absorbance at 23.8 kK had disappeared. Under these conditions the time required for inactivation was 5-6 hours. The inactivated enzyme was then dialyzed overnight against a two liter volume of 0.1 M acetate, pH 4.6 with two changes. The dialyzed material was then concentrated by ultrafiltration using an Amicon PM 10 membrane to approximately 10 ml. Twenty ml of glass distilled water were added and the solution reconcentrated to 10 ml. The pH of the concentrate was adjusted to 11 and the solution turned a bright yellow. After stirring
for one hour at 25°C, \( \text{I} \) was separated from the protein by ultrafiltration. To remove \( \text{Na}^+ \) the yellow filtrate was applied to a 0.7 x 2 cm column of Dowex 50 (H\(^+\) form), 100-200 mesh, and eluted with distilled water. After lyophilization the resulting yellow powder was dissolved in 0.2 ml of water and further purified by high voltage electrophoresis. The support employed was a 20 x 40 cm sheet of Whatman 3 mm paper; the electrolyte, 0.1 M acetate, pH 4.7. Electrophoresis was carried for 20 minutes at 2000 V. Under these conditions \( \text{I} \) had the greatest electrophoretic mobility, 6.5 cm. The sample could be located by spraying a test strip of the electropherogram with Gibbs reagent or by its distinct orange fluorescence when illuminated by a Mineralight UVS-11 (Ultraviolet Products Inc., San Gabriel, Calif.). \( \text{I} \) was eluted from the dried electropherogram with water. The eluate was then applied to a 0.7 x 1 cm column of Dowex 50 (H\(^+\) form) 100-200 mesh and eluted with water and lyophilized.

**Dephosphorylation of \( \text{I} \)**

A solution of \( \text{I} \) obtained by ultrafiltration of the inactivated enzyme after treatment with base was acidified to pH 8.1 with 0.05 N HCl. To this was added approximately 0.03 mg of \( \text{E. coli} \) alkaline phosphatase (code HAPF, Worthington Biochemical). The reaction was followed by the decrease in absorbance at 24.6 kK. After approximately 13 hours of stirring at room temperature there were no further changes observed in the spectrum. The solution was lyophilized and then dissolved in 0.3 ml of water and purified by high voltage electrophoresis. The support employed was a 20 x 40 cm sheet of Whatman 3 mm paper; the electrolyte, 0.1 M acetic acid adjusted to pH 4.7 with pyridine. Electrophoresis was carried
out for 20 minutes at 2000 V. Under these conditions the major product (as determined qualitatively by the intensity of the Gibbs test) migrated 2.5 cm toward the anode. After drying the electropherogram, the material was eluted with water and lyophilized. A spectrum of the material showed an absorption band at 32.1 kK and a shoulder at 40 kK at pH 5.8.
RESULTS

Interaction of ApoGAD with 5' Analogs of PLP

The structures of the pyridoxal phosphate analogs used in this study are indicated below.

\[
\begin{align*}
R & \quad \text{Pyridoxal-5'-phosphate} \\
\text{I} & \quad \text{trans-5-Nor-}\beta\text{-carboxyvinylpyridoxal} \\
\text{II} & \quad \text{5-Nor-5-}\beta\text{-carboxyethylpyridoxal} \\
\text{III} & \quad \text{trans-5-Nor-5-}\beta\text{-vinylpyridoxal phosphonate} \\
\text{IV} & \quad \text{5-Nor-5-}\beta\text{-ethylpyridoxal phosphonate} \\
\text{V} & \quad \text{Pyridoxal-5'-sulfonate} \\
\text{VI} & \quad \text{Pyridoxal-5'-sulfate}
\end{align*}
\]

Reconstitution of apoGAD with PLP

Addition of PLP (4.01 x 10^{-5} M) to apoGAD (5.84 x 10^{-5} M) resulted in an increase in absorbance at 23.8 kK. The spectrum became constant after one hour. The spectrum of the reconstituted enzyme is shown in Figure 6. Comparison of the 'fine structure' derived from the difference of the experimental curve and a fitted lognormal curve (Metzler et al., 1973) for both the native and reconstituted enzyme (Figure 7) indicated that the coenzyme is rebound to the protein in the same manner as it is bound in the native enzyme. Typically the apoenzyme could be reactivated to 80-90%
Figure 6. ApoGAD reconstituted with PLP; [apoGAD], $5.84 \times 10^{-5}$ M; PLP, $4.01 \times 10^{-5}$ M. The protein absorbance has been subtracted out.
Figure 7. Fine structure plots for (A) native enzyme and (B) reconstituted enzyme
of its original activity. The CD spectrum of the reconstituted enzyme is shown in Figure 8.

**Carboxylic acid analogs (I and II)**

Addition of I (5-nor-5-β-carboxyvinylpyridoxal) to apoGAD resulted in the formation of an absorption band at 23.0 kK (Figure 9) with +CD at 23.2 kK and a -CD band at 31 kK (Figure 10). The complex was devoid of any measurable activity. Glutamate at a concentration of 1 mM had no effect on the spectrum or CD of the apo-analog complex.

Reduction of the apoGAD–I complex with NaBH₄ led to the formation of a band at 29.5 kK that exhibited a weak +CD band at 20.0 kK.

Addition of an excess of hydroxylamine to the apoGAD–I complex resulted in a decrease of the band at 23.0 kK and appearance of a band at 29.2 kK. The rate of disappearance of the 23.0 kK absorption band followed first order kinetics. The rate of oxime formation was inhibited by glutamate.

Preliminary results of the inhibition of oxime formation by glutamate indicate the kinetic mechanism shown below. The data are summarized in Table 2.

$$\begin{align*}
\text{ApoGAD–I} & \xrightarrow{k_1'} \text{oxime} \\
+ & \\
\text{glutamate} & \\
K & \\
\text{ApoGAD–I–glutamate}
\end{align*}$$
Figure 8. CD spectrum of apoGAD reconstituted with PLP
Figure 9. Binding of I to apoGAD: A) apoGAD, $6.6 \times 10^{-5}$ M; B) apoGAD, $6.53 \times 10^{-5}$ M, I, $6.46 \times 10^{-5}$ M, 3 min. after addition of analog; C) 69 min.; D) 126 min.; E) 448 min.; F) 1738 min.
Figure 10. A) CD spectrum of apoGAD-I complex, $2.51 \times 10^{-5}$ M; B) reduced apoGAD-I.
Table 2. Summary of rate data for reaction of NH₂OH with apoGAD-I (measurements were made using two different preparations of apoGAD-I)

<table>
<thead>
<tr>
<th>[Glu] mM</th>
<th>$k_{obs}$ (sec⁻¹)</th>
<th>$K$ (mM)</th>
<th>$k$ (mM⁻¹ sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.56 x 10 (k₁')</td>
<td>-</td>
<td>4.60 x 10⁻⁴</td>
</tr>
<tr>
<td>5</td>
<td>2.32 x 10</td>
<td>48.3</td>
<td>4.61 x 10⁻⁴</td>
</tr>
<tr>
<td>10</td>
<td>2.09 x 10</td>
<td>44.5</td>
<td>4.54 x 10⁻⁴</td>
</tr>
<tr>
<td>0</td>
<td>6.84 x 10 (k₁')</td>
<td>-</td>
<td>4.75 x 10⁻⁴</td>
</tr>
<tr>
<td>75</td>
<td>2.80 x 10</td>
<td>51.8</td>
<td>4.97 x 10⁻⁴</td>
</tr>
<tr>
<td>Avg</td>
<td>48.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Calculated (see text).

^b NH₂OH, 5.56 mM; initial absorbance of apoGAD-I at 23.0 kK = 0.260.

^c NH₂OH, 1.44 mM; initial absorbance of apoGAD-I at 23.0 kK = 0.275.
Rate = \( k'_1 [\text{ApoGAD-I}] \)

where \( k'_1 = k[\text{NH}_2\text{OH}] \) 

\[
\text{Rate} = \frac{K k'_1 [\text{ApoGAD-I}]}{K + [\text{glutamate}]} 
\]

where \( K = \frac{[\text{ApoGAD-I}][\text{glutamate}]}{[\text{ApoGAD-I-glutamate}]} \) and \( [\text{ApoGAD-I} \_t] = [\text{ApoGAD-I}] + [\text{ApoGAD-I-glutamate}] \)

\[
k_{obs} = \frac{K k'_1}{K + [\text{glutamate}]} 
\]

or \( K = \frac{k_{obs} [\text{glutamate}]}{k'_1 - k_{obs}} \) 

substituting (1) for \( k'_1 \) and rearranging

\[
k = \frac{k_{obs} [K + \text{glutamate}]}{K[\text{NH}_2\text{OH}]} . \tag{3}
\]

From the measured values of \( k_{obs} \) and \( k'_1 \) (\( k'_1 \) is the observed rate constant for oxime formation in the absence of glutamate), the dissociation constant (\( K \)) for glutamate could be calculated (Table 2) using equation 2. Using the average value of \( K \) and the data in Table 2, \( k \) was calculated for each set of conditions using equation 3. The consistency of the value of \( k \) suggested that the mechanism was most likely correct. Although more data are needed, it appeared that the reaction of apoGAD-I with hydroxylamine was either a bimolecular reaction or one involving a steady state intermediate, both of which are kinetically indistinguishable.

Addition of II (5-nor-5-\( \beta \)-carboxyethylpyridoxal) to apoGAD resulted in the formation of an absorption band at 23.8 \( \text{kK} \) (Figure 11). The bound
Figure 11. Binding of II to apoGAD: A) apoGAD, $6.85 \times 10^{-5}$ M; B) apoGAD, $6.81 \times 10^{-5}$ M, II, $6.13 \times 10^{-5}$ M, 4 min. after addition of analog; C) 55 min.; D) 110 min.; E 327 min.; F) 1225 min.
analog exhibited +CD at 23.9 kK (Figure 12). Addition of glutamate to a concentration of 2.8 mM had no effect on the spectrum or on the CD of the complex. After 12.5 hours of incubation with glutamate the spectrum remained unchanged. The analog did not reactivate the apoenzyme to any measurable extent.

Reaction of the apo-analog complex with hydroxylamine led to the formation of a band absorbing at 30.8 kK.

**Phosphonate analogs (III and IV)**

Addition of III (5-nor-5-β-vinylpyridoxal phosphonate) to apoGAD resulted in the formation of absorbance bands at 23.4 kK and 30.2 kK (Figure 13). The spectrum became constant after 107 minutes. The absorbance band at lower energy is optically active (Figure 14). Addition of L-glutamate resulted in an immediate diminution of the +CD at 23.2 kK with a concomitant appearance of a -CD band at approximately 27.8 kK. These immediate changes are reversed although not completely. The +CD at lower energy does not completely return and in addition a small +CD band is observed at approximately 30.3 kK (Figure 15).

Accompanying the changes in CD the following absorbance changes were noted (Figure 16). Within three minutes after addition of glutamate the absorbance at 23.4 kK drops to approximately half of its original value with a concomitant increase in absorbance at 26.7 kK and decrease at 30.2 kK. With time these changes are partially reversed. After 111.5 minutes no further changes were seen in the spectrum. The absorbance at 30.2 kK returned to its original value, whereas the approximately 75% of the original absorbance at 23.4 kK returned. A definite shoulder can be seen
Figure 12. CD spectrum of apoGAD-II; apoGAD, $3.89 \times 10^{-5}$ M, II, $3.49 \times 10^{-5}$ M
Figure 13. Binding of III to apoGAD: A) apoGAD, $1.54 \times 10^{-4}$ M; B) apoGAD, $1.48 \times 10^{-4}$ M, III, $1.27 \times 10^{-4}$ M, 4 min. after addition of analog; C) 16 min.; D) 50 min.; E) 182 min.
Figure 14. CD spectrum of apoGAD-III complex; A) apoGAD, $1.48 \times 10^{-4}$ M, III, $1.27 \times 10^{-5}$ M; B) apoGAD-III reduced by NaBH₄.
Figure 15. Changes in CD spectrum upon addition of glutamate to apoGAD-III: A) apoGAD-III; B) 13 min. after addition of glutamate; C) 40 min.; D) 99.5 min. Conditions same as for Figure 16.
Figure 16. Reaction of apoGAD-III with glutamate: A) apoGAD, 1.48 x 10^{-4} M, III, 1.27 x 10^{-4} M; B) 3 min. after addition of glutamate; C) 28.5 min.; D) 49 min.; E) 111.5 min., apoGAD, 1.46 x 10^{-4} M, III, 1.25 x 10^{-4} M, glutamate, 6.17 mM
at 26.7 kK. The ratio of $\Delta A/A = 4.75 \cdot 10^{-4}$ prior to the addition of glutamate. After incubation of the apoGAD-III complex with glutamate for 40 hours, $\Delta A/A = 4.84 \cdot 10^{-4}$.

Reduction with NaCNBH$_3$ of the initial apoGAD-III complex, the "26.7 kK intermediate," and the complex (complexes?) after 40 hours of incubation with glutamate yielded the same species characterized by an absorption band at 29.1 kK (Figure 17) and a small +CD band at 28.6 kK (Figure 14). In order to determine whether or not the analog was bound to the protein, the protein was precipitated with trichloroacetic acid. Upon redissolving the precipitates in 6 M guanidine HCl, it was found that the majority of the analog had remained bound to the protein in all three complexes.

Titration of the apoGAD-III complex indicated that the absorption bands at 23.4 and 30.2 kK were pH related as in the case for the native enzyme (Shukuya and Schwert, 1960). Lowering the pH from 4.6 resulted in an increase in absorbance at 23.6 kK and a decrease at 30.2 kK; raising the pH from 4.6 gave a decrease at 23.6 kK and an increase at 30.2 kK.

The absorption and CD spectra of the apoGAD-IV complex are shown in Figures 18 and 19. Addition of IV to the apoenzyme resulted in the formation of an absorption band at 24.3 kK with a shoulder at 31 kK. Addition of glutamate (final concentration, 6 mM) had no effect on the absorption spectrum.

Reduction of the apoGAD-IV complex with NaCNBH$_3$ resulted in a decrease of absorbance at 24.5 kK yielding a final spectrum with a distinct shoulder at approximately 26 kK and a peak at 30.4 kK.
Figure 17. A) apoGAD-III; B) "26.7 kK intermediate" obtained upon addition of glutamate; C) and D) NaBH₄ reduction products of A and B respectively.
Figure 18. ApoGAD reconstituted with IV: A) apoGAD, $1.58 \times 10^{-4}$ M, B) apoGAD, $1.52 \times 10^{-4}$ M, IV, $1.33 \times 10^{-4}$ M
Figure 19. CD spectrum of apoGAD-IV complex; apoGAD, $1.52 \times 10^{-4}$ M, IV, $1.33 \times 10^{-4}$ M
Both analogs failed to reactivate apoGAD to any measurable extent, even though glutamate appears to interact with the apoGAD-III complex.

Sulfonate analog (V)

Addition of V (pyridoxal-5'-sulfonate) to apoGAD resulted in the formation of an absorption band at 23.9 kK (Figure 20). The apo-analog complex exhibited +CD at 23.9 kK (Figure 21). The complex was devoid of any measurable catalytic activity. Reaction of the apoGAD-V complex with hydroxylamine led to the formation of a band absorbing at 30.9 kK.

Sulfate analog (VI)

Addition of VI (pyridoxal-5'-sulfate) to apoGAD resulted in the formation of an absorption band at 24.4 kK (Figure 22) and exhibited a +CD band at 24.1 kK. Addition of glutamate resulted in a decrease in the CD at 24.1 kK and in the appearance of a small -CD band at 27.4 kK. With time these changes partially reversed as shown in Figure 23. Approximately 50% of the +CD at 24.1 kK returned after 45 minutes. The absorption spectrum after 45 minutes showed that the absorbance at 24.5 kK was approximately 45% of that before the addition of glutamate. Although turbidity precluded further measurement it appears that the apoGAD-VI complex interacts with glutamate although no measurable reactivation of the apoenzyme could be detected.

Summary

The spectral properties of the apoGAD-analog complexes are summarized in Table 3. All appeared to bind to the protein as evidenced by the bathochromic shift in the spectrum of the analog upon incubation with
Figure 20. Binding of V to apoGAD: A) apoGAD; B) 8 min. after addition of analog; C) 33 min.; D) 80 min.; E) 140 min.; F) 673 min.
Figure 21. CD spectrum of apoGAD-V complex, 6.92 x 10^{-5} M
Figure 22. Binding of VI to apoGAD: A) apoGAD, $5.82 \times 10^{-5}$ M, B) apoGAD, $5.78 \times 10^{-5}$ M, VI, $5.60 \times 10^{-5}$ M, 2 min. after addition of analog; C) 34 min.; D) 150 min.
Figure 23. CD spectrum of apoGAD-VI complex; A) apoGAD-VI, \(5.6 \times 10^{-5}\) M, B) 20 min. after addition of glutamate; C) 45 min. 4.9 \(\times 10^{-5}\) M apoGAD-VI, 12.4 mM glutamate
Table 3. Summary of band positions for the apoGAD-analog complexes

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{max}}$</th>
<th>$\lambda_{\text{CD}}$</th>
<th>$\Delta\varepsilon/\varepsilon$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>23.8 kK</td>
<td>23.8 kK</td>
<td>$1.28 \times 10^{-3}$</td>
</tr>
<tr>
<td>I</td>
<td>23.0 kK</td>
<td>23.2 kK</td>
<td>$1.17 \times 10^{-3}$</td>
</tr>
<tr>
<td>II</td>
<td>23.8 kK</td>
<td>23.9 kK</td>
<td>$1.36 \times 10^{-3}$</td>
</tr>
<tr>
<td>III</td>
<td>23.4 kK</td>
<td>23.2 kK</td>
<td>$0.43 \times 10^{-3}$</td>
</tr>
<tr>
<td>IV</td>
<td>24.3 kK</td>
<td>23.9 kK</td>
<td>$0.36 \times 10^{-3}$</td>
</tr>
<tr>
<td>V</td>
<td>23.9 kK</td>
<td>23.9 kK</td>
<td>$1.33 \times 10^{-3}$</td>
</tr>
<tr>
<td>VI</td>
<td>24.4 kK</td>
<td>24.1 kK</td>
<td>$0.47 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
apoenzyme and the appearance of CD. All of the analogs tested failed to reactivate the apoenzyme.

**Reaction of GAD with Substrate Analogs**

**Reaction of GAD with the phosphonate analog of glutamate**

The γ phosphonate analog of glutamate does not react with GAD. Incubation of the enzyme (6.1 x 10^{-5} M) with 10 mM analog in 0.1 M pyridine HCl, pH 4.6, did not affect the spectrum after 12 hours of incubation. The analog is not active as a substrate. CO₂ was not detected manometrically upon addition of 1.5 µ-moles of GAD to a 16.7 mM solution of the analog in 0.1 M pyridine HCl, pH 4.6. Furthermore no inhibition of decarboxylase activity was observed at a concentration of 12.5 mM analog when assayed using a 5 mM solution of glutamate in 0.1 M pyridine HCl, pH 4.6, 0.15 M NaCl and 0.15 µ-mole of enzyme. It appears that the analog did not interact with the enzyme.

**Inactivation of GAD by L-serine-O-sulfate**

Incubation of GAD with serine sulfate in 0.05 M sodium acetate, pH 4.6, led to a decrease in absorbance of the holoenzyme at 23.8 kK and an increase in absorbance at 29.8 kK (Figure 14) as previously demonstrated by Sukhareva and Braunstein (1971). CO₂ was not detected manometrically upon addition of the analog to GAD. Concomitant with the spectral changes was the loss of enzymic activity (Figure 25). After 90 minutes of incubation with serine sulfate less than 10% of the activity remained. The enzyme is totally inactivated after 4 hours of incubation. The inactivated enzyme is devoid of optical activity in the region of 20–33 kK, whereas
Figure 24. Reaction of GAD with L-serine-O-sulfate: A) holoenzyme, 4.05 x 10^{-5} M; B) 1.4 min. after addition of serine sulfate (12.5 mM); C) 6.5 min.; D) 13.5 min.; E) 23.2 min.; F) 39.2 min.; G) 62.0 min.; H) 123.9 min. 0.05 M sodium acetate, pH 4.6; 10°C
Figure 25. Inactivation of GAD by L-serine-O-sulfate. Conditions; same as for Figure 24; 6 μl aliquots of the reaction mixture was assayed for decarboxylase activity in the absence (●●) and in the presence of serine sulfate (○○); the amount of active GAD remaining as determined from the absorbance at 23.8 kK is also plotted (x-x)
the active enzyme is characterized by a +CD band at 23.8 kK.

The inactivation followed first order kinetics as shown in Figure 26. The curvature in the plot is indicative of two parallel first order reactions that produce a common product. The plot was curved irrespective of the manner in which the data were treated, that is, as a first or second order reaction. The observed rate constant for the slower reaction was determined to be 0.029 min⁻¹. The rate constant for the faster reaction, which can only be estimated due to a lack of data points during the early stages of the reaction, is approximately 0.12 min⁻¹. It is estimated that the slower reaction accounts for approximately 40% of the total absorbance change.

In order to determine the kinetic mechanism, the rate of reaction was studied as a function of serine sulfate concentration. In contrast to the reaction done in acetate buffer, the first order plots were linear (Figure 27) when the reaction was done in 0.10 M pyridine HCl, pH 4.6. A plot of the reciprocal of the observed rate constants (obtained from the decrease in absorbance at 23.8 kK) versus the reciprocal of the serine sulfate concentration is linear with a finite Y intercept (Figure 28). A minimal kinetic mechanism consistent with these data is shown below.

\[
\begin{align*}
\text{K} + \text{SerSO}_4 & \rightarrow \text{[X]} \rightarrow \text{inactivated GAD} \\
\text{Rate} & = k[X] \\
K & = \frac{[\text{GAD}][\text{SerSO}_4]}{[X]} \\
& = \frac{k[\text{SerSO}_4][\text{GAD}]}{K}
\end{align*}
\]
Figure 26. Time course of GAD inactivation by L-serine-O-sulfate in 0.05 M acetate, pH 4.6. [GAD], $4.1 \times 10^{-5}$ M; [serine sulfate], 12.5 mM; 10°C
Figure 27. Time course of GAD inactivation by L-serine-O-sulfate in 0.1 M pyridine HCl, pH 4.6. [GAD], 4.0 x 10^{-5} M [serine sulfate], 50 mM; 10°C
Figure 28. Double reciprocal plot of GAD inactivation by L-serine-O-sulfate in 0.1 M pyridine HCl, pH 4.6. [GAD], 4.1 x 10^{-5} M; 10^\circ C
where \([\text{GAD}]^e = [\text{GAD}] + [X]\)

\[
\frac{K_k[\text{SerSO}_4][\text{GAD}^e]}{K + \text{SerSO}_4}
\]

\[
k_{\text{obs}} = \frac{K_k[\text{SerSO}_4]}{K + [\text{SerSO}_4]}
\]

The data are summarized in Table 4. The kinetic parameters obtained from the intercept and slope are; \(k_2 = 0.036 \text{ min}^{-1}\) and \(K = 11.5 \text{ mM}\).

In 0.1 M pyridine \(\text{SO}_4\), pH 4.6, the first order plots for inactivation were biphasic. The data obtained at various serine sulfate concentrations (Figure 29) were consistent with the postulated kinetic mechanism. The kinetic parameters obtained are: for the slow reaction, \(k_2 = 5.09 \times 10^{-3} \text{ min}^{-1}\) and \(K = 12.1 \text{ mM}\); for the fast reaction, \(k_2 = 0.016 \text{ min}^{-1}\) and \(K = 7.6 \text{ mM}\). The data are summarized in Table 5.

D,L-serine-0-phosphate appeared to react in a manner analogous to serine sulfate. Addition of serine phosphate (12.5 mM) to the holoenzyme (3.4 \(\times 10^{-5}\) M) resulted in the formation of a band at 29.8 kK. The reaction with serine phosphate was much slower than that with serine sulfate. Approximately 750 minutes were required to lose 50% of the initial absorbance at 23.8 kK, whereas approximately 4 minutes were required when serine sulfate was used.

**Chemical and physical properties of inactivated GAD**

Upon examination of the products of the inactivation reaction, it was found that the absorbance at 29.8 kK (335 nm) was not due to FMP. Precipitation of the inactivated enzyme with trichloroacetic acid and analysis of the supernatant indicated that the chromophore remained with the
Figure 29. Double reciprocal plot of GAD inactivation by L-serine-O-sulfate in 0.1 M pyridine $\text{SO}_4$, pH 4.6. [GAD], $4.0 \times 10^{-5}$ M; 10°C
Table 4. Summary of rate data for inactivation of GAD by serine sulfate in pyridine HCl, pH 4.6\textsuperscript{a}

<table>
<thead>
<tr>
<th>SerSO\textsubscript{4} (mM)</th>
<th>$k_{\text{obs}}$ (min\textsuperscript{-1})\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.00</td>
<td>0.0279</td>
</tr>
<tr>
<td>16.66</td>
<td>0.0233</td>
</tr>
<tr>
<td>10.00</td>
<td>0.0171</td>
</tr>
<tr>
<td>7.15</td>
<td>0.0135</td>
</tr>
</tbody>
</table>

\textsuperscript{a}GAD, $4.0 \times 10^{-5}$ M; buffer, 0.1 M pyridine HCl, pH 4.6.

\textsuperscript{b}$k_{\text{obs}}$ was determined from a linear least squares fit of the data as described in Experimental. Temperature = 10°C.
Table 5. Summary of rate data for inactivation of GAD by serine sulfate in pyridine SO₄, pH 4.6

<table>
<thead>
<tr>
<th>SerSO₄ (mM)</th>
<th>$k_{obs}^{\text{slow}}$ (min⁻¹)</th>
<th>$k_{obs}^{\text{fast}}$ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40.0</td>
<td>$3.83 \times 10^{-3}$</td>
<td>$1.37 \times 10^{-2}$</td>
</tr>
<tr>
<td>20.0</td>
<td>$3.26 \times 10^{-3}$</td>
<td>$1.07 \times 10^{-2}$</td>
</tr>
<tr>
<td>13.3</td>
<td>$2.68 \times 10^{-3}$</td>
<td>$1.03 \times 10^{-2}$</td>
</tr>
<tr>
<td>10.0</td>
<td>$2.29 \times 10^{-3}$</td>
<td>$0.89 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

\(^a\)GAD, $4.0 \times 10^{-5}$ M; buffer, 0.1 M pyridine SO₄, pH 4.6.

\(^b\) $k_{obs}^{\text{slow}}$ is the observed rate constant for the linear portion of the reaction, $k_{obs}^{\text{fast}}$ was calculated as described in Experimental. Both rate constants were determined from a linear least squares fit. Temperature = 10°C.
protein, whereas the reaction with $\alpha$-methylglutamate yields a final product that absorbs at 30.8 kK (325 nm) which is easily separated from the protein and has been identified as PMP (Huntley and Metzler, 1968).

Dialysis of the reaction mixture against 0.05 M acetate, pH 4.6, for 24 hours did not alter the band position of the inactivated enzyme. The $A_{35.5\text{kK}}/A_{29.8\text{kK}}$ ratio was identical for the inactivated enzyme before and after dialysis indicating that the chromophore remained bound to the protein during dialysis. Assuming that the extinction coefficient at 35.5 kK of the inactivated enzyme is the same as that for the holoenzyme (88,000 M$^{-1}$cm$^{-1}$; Fonda, 1971), the extinction at 29.8 kK is estimated to be approximately 8200 M$^{-1}$cm$^{-1}$. This value closely agrees with the value of 8500 M$^{-1}$cm$^{-1}$ which is based upon the extinction of the active enzyme at 23.8 kK.

Addition of PLP did not reactivate the modified enzyme.

The possibility existed that the absorbance at 29.8 kK could be due to the neutral tautomer of the PLP-enzyme Schiff base which has been demonstrated to exist in a nonpolar environment (Heinert and Martell, 1963a, 1963b) and is thought to be the structure responsible for the absorption band in phosphorylase b (Johnson et al., 1970). Denaturation of the protein then should lead to a bathochromic shift upon exposure of the Schiff base to solvent. Furthermore if the 29.8 kK species is a neutral Schiff base, PLP should be released from the protein upon raising the pH.

Dialysis to the inactivated enzyme against 8 M urea, 0.05 M acetate, pH 4.6, overnight led to a small hypsochromic shift (from 29.8 kK to approximately 30.5 kK) and a 50% decrease in the extinction of the low
energy band when calculated relative to the protein extinction at 35.5 kK. The reason for this decrease is not clear but it is not due to release of the chromophore (see below). PLP was not formed upon raising the pH.

The position of the absorption band of the inactivated enzyme is not affected by addition of excess sodium borohydride or sodium cyanoborohydride at pH 4.6 (Figure 30).

However if the pH of the inactivated enzyme is raised to 10, there is a large bathochromic shift to 23.8 kK (Figure 31). Furthermore the chromophore responsible for the yellow color can be separated from the protein either by precipitation of the protein with trichloroacetic acid or by ultrafiltration. This material is designated as 1 (see below). For comparison raising the pH of the holoenzyme leads to a hypsochromic shift to 25.8 kK yielding free PLP.

Denaturation of the inactivated enzyme with 10 M urea and precipitation of the protein with trichloroacetic acid did not result in release of a 3-OH pyridine compound. Incubation of the inactivated enzyme in 10 M urea, pH 1.5, at 35°C for 24 hours did not result in release of the chromophore. In comparison precipitation of the active enzyme with trichloroacetic acid is sufficient to hydrolyze 35% of the covalently bound PLP.

Although it would appear that treatment of the inactivated enzyme with reducing agents or acid has no effect, this is not the case. If the pH of a solution containing the inactivated enzyme is made alkaline (pH 11-12) after treatment with borohydride or acid, the absorbance is shifted to higher energies (presumably under the protein band) and more
Figure 30. Reduced inactivated GAD: A) pH 4.6; B) pH 11
Figure 31. Inactivated GAD: A) pH 4.6; B) pH 11
importantly formation of the yellow species does not occur nor is there release of a 3-OH pyridine compound.

It appeared to be more difficult to reduce the inactivated enzyme than the holoenzyme. Even though there is extensive hydrolysis of NaBH₄ at pH 4.6, the active enzyme was reduced immediately upon addition of borohydride, whereas after 15 min of incubation with borohydride the inactivated enzyme was not fully reduced as evidence by the appearance of the yellow species upon addition of base. Although sodium cyanoborohydride is more stable at pH 4.6 several hours appeared to be required for the reduction of the inactivated enzyme to go to completion. For the reduced inactivated enzyme in 6 M guanidine HCl, pH 6.2, there is an absorption band at approximately 30.7 kK.

It is evident that the enzyme had been covalently modified upon reaction with serine sulfate. It was clear that the cofactor was not bound to the protein through the usual Schiff base linkage or as a simple ketimine. Hydrolysis of the former would yield a pyridoxal derivative, whereas hydrolysis of the latter would yield a pyridoxamine derivative. Characterization of 1 should give a clue as to what has occurred during the reaction of GAD with serine sulfate.

Chemical and physical properties of 1

Spectrophotometric titration of the material isolated by ultrafiltration of the inactivated enzyme after addition of NaOH (pH 11) indicated pK's of 8.74 and 3.93 (Figure 32). Titration of 1 that had been further purified (see Experimental) gave similar results (Figure 33). The spectral changes observed as a function of pH are shown in Figure 34. The tight
Figure 32. Absorbance of 1 at 22.0 (x-x) and 25.0 (o-o) plotted vs. pH. The calculated curves are based upon pK values of 3.93 and 8.74.
Figure 33. Absorbance of $\text{1}$ at 22.0 (x–x), 25.0 (o–o), and 34.0 (↑↑) plotted vs. pH. The calculated curves are based on the spectra in Figure 34 and pK values of 3.98 and 8.69.
Figure 34. Spectra of $\frac{1}{\text{H}}$ at pH 2.3 (A), 4.1 (B), 6.1 (C), 8.7 (D), and 10.7 (E)
Isosbestic points indicated that the material was relatively pure and free of contaminating material absorbing in this region of the spectrum. The effect of pH on the spectrum is completely reversible. The calculated ionic forms of $1$ are shown in Figure 35.

The material reacted with Gibbs reagent (N,2,6-trichloro-p-benzoquinoneimine) to give the characteristic blue color indicative of a phenolic function para to an aromatic hydrogen (Gibbs, 1927).

It was immediately apparent that $1$ was not PMP or a derivative with a saturated 4' carbon atom. Characteristic band positions for these derivatives are 34.0-34.7 kK for the cationic forms; 30.0-31.6 kK for the dipolar ionic forms and 31.6-32.6 kK for the anionic forms (Metzler et al., 1973). Furthermore $1$ did not give the distinctive orange color upon reaction with ninhydrin as does PMP and PM.

Comparison of the band positions of $1$ to those of $B_6$ aldehydes indicated that $1$ was not an aldehyde. The most anionic (P) form of $1$ absorbed at 23.5 kK, whereas the anionic aldehydic forms of $B_6$ and its derivatives absorb at 25.5-25.7 kK (Harris et al., 1976).

$1$ was not an acid derivative. In 0.1 M NaOH 4-pyridoxic acid absorbs at 32.5 kK (Burg et al., 1960). Additionally pyridoxic acid exhibits a bright blue fluorescence, whereas $1$ fluoresces with an orange color.

The low energy absorption of $1$ suggested that it was a Schiff base derivative. Typically the lowest energy bands of Schiff bases (23.0-23.8 kK) are bathochromically shifted from those of the free aldehydes (25.5-26.0 kK).
Figure 35. Ionic forms of $\text{P} (A)$, $\text{HP} (B)$, and $\text{H}_2\text{P} (C)$. Calculated spectra are based on the spectra in Figure 34 and pK values of 3.98 and 8.69.
In general protonation of a Schiff base on the ring nitrogen (pK 6-6.5) has only a small effect on the spectrum. However dissociation of the proton from the imino nitrogen leads to a strong hypsochromic shift to about 27.0-27.5 kK.

Heinert and Martell (1963a, 1963b) have shown that the enamine (I, Figure 36) rather than the tautomeric phenolic (III) structure is responsible for the low energy absorption. In fact there appears to be a correlation between the observed band positions and the electron donating ability of the phenolic group and the electron accepting ability of the substituent on the 4 carbon of the pyridine ring (Johnson and Metzler, 1970). Thus the lowest energy bands are found for those Schiff bases in which the phenolic group is ionized and the imino nitrogen protonated (Figure 36).

Although the band positions for the ionic forms of 1 suggest that it is a Schiff base, three differences between 1 and Schiff bases of the type shown in Figure 36 should be noted. 1 is unusually stable at high pH (13) whereas extensive hydrolysis of the valine-PLF Schiff base was observed at pH 11. Furthermore the large hypsochromic shift indicative of dissociation of the imino proton was not observed for 1, suggesting that the imino nitrogen was disubstituted. Lastly the observed pK's of 1 differed from those of the usual Schiff base. The small change in the spectrum between pH 6-10 suggested that the pK of 8.74 was that of the ring nitrogen and the pK of 3.9 was that of the phenolic group. The pK's of 1 are approximately 2 pH units higher than the corresponding ones of Schiff bases. For comparison the pK's of 1 are within the ranges of pK values for the B6 aldehyde derivatives (Harris et al., 1976) and for pyridoxamine and
Figure 36. Ionic and tautomeric forms of PLP-Schiff base derivatives
pyridoxine derivatives (Metzler et al., 1973). Any proposed structure for 1 must account for these differences.

High voltage electrophoresis of 1 at different pH values indicated that the net charge on the molecule was negative at all pH values studied suggesting that the phosphate group was still present (Figure 37, Table 6). The possibility that the strong acidic group was the sulfate could be ruled out. β-Chloroalanine reacted in the same manner with the enzyme yielding an inactive enzyme absorbing at 29.8 kK. Addition of base resulted in the formation of a compound with spectral and electrophoretic properties identical to those of 1. Although it is conceivable that substitution of a chloro group for a sulfate may have no effect on the spectral properties, there would be a marked difference in the electrophoretic properties if 1 did indeed have a sulfate group and the material isolated from the β-chloroalanine reaction contained a chloro group. It seemed likely that either during the initial inactivation reaction or during the generation of 1, the sulfate (chloro) group was eliminated.

It is of interest to note that the mobility of 1 is identical at pH 6.5 and 4.7 (Figure 37, Table 6), whereas the mobilities of PLP and PMP decrease due to protonation of the phosphate which for PLP has a pK_2 of 6.1 (Harris et al., 1976) and for PMP a pK_2 of 5.76 (Metzler et al., 1973). This apparent "loss" of the second pK of the phosphate group of 1 suggested that 1 contained either a phosphodiester or that the phosphate was interacting with a positively charged basic group on the molecule.

The presence of the phosphate group in 1 could be demonstrated by the decrease in electrophoretic mobility of 1 (Figure 37, Table 6) and the
Figure 37. Electrophoresis of \( \text{L} \) and its derivatives. (1), PLP; (2), PMP; (4), PM; (5), \( \text{L} \); (6), basic hydrolysis product of \( \text{L} \); (7), dephosphorylated \( \text{L} \). Conditions: 2000 V for 15 min. Negative migration is toward the cathode. Buffers are given in Table 6.
Table 6. Summary of electrophoretic data for 1 and its derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 10^a</th>
<th>Migration relative to PLP</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.5^b</td>
<td>pH 4.7^c</td>
<td>pH 3.3^d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLP</td>
<td>1.00 (-3.00)^e</td>
<td>1.00 (-1.73)</td>
<td>1.00 (-0.96)</td>
<td>1.00 (-0.32)</td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>0.39±0.01 (-1.00)</td>
<td>-0.05^f (+0.04)</td>
<td>-0.36±0.04 (+0.25)</td>
<td>-5.75 (+0.89)</td>
<td></td>
</tr>
<tr>
<td>PMP</td>
<td>0.68±0.02 (-2.20)</td>
<td>0.46±0.05 (-0.86)</td>
<td>0.08±0.01 (-0.04)</td>
<td>-2.62 (+0.53)</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>0.07 (-0.20)</td>
<td>-1.05±0.04 (+0.98)</td>
<td>-1.44 (+1.05)</td>
<td>-8.75 (+1.58)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.04±0.02</td>
<td>1.31±0.01</td>
<td>1.69±0.02</td>
<td>3.50</td>
<td></td>
</tr>
<tr>
<td>1 AP^g</td>
<td>0.46</td>
<td>0.65±0.03</td>
<td>0.57±0.01</td>
<td>-1.06</td>
<td></td>
</tr>
<tr>
<td>1 BH^h</td>
<td>0.98±0.02</td>
<td>1.04±0.02</td>
<td>1.04±0.07</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

^a0.05 M NaHCO₃.
^b0.1 M sodium cacodylate.
^c0.1 M sodium acetate.
^d0.4 M sodium formate.
^eThe number in parentheses indicates the net charge on the molecule at that pH.
^fNegative values indicate migration toward the cathode.
^gAlkaline phosphatase treated 1.
^hBasic hydrolytic product of 1.
detection of ortho phosphate on the electropherogram after treatment with
*E. coli* alkaline phosphatase (see Experimental).

In addition to the change in electrophoretic mobility, dephosphoryla-
tion of 1 at pH 8.1 led to a decrease in the absorbance at 24.2 kK and an
increase in absorbance at 32.2 kK (Figure 38). Titration of the dephos-
phorylated material gave spectral changes similar to those seen for the
titration of 1 (Figure 39) with the notable exception, that in addition
to the presence of a band at 32.2 kK at pH 8, the intensity of the low
energy bands was greatly decreased. This suggested that the phosphate
group was stabilizing the structure responsible for the low energy absorb-
ance. A pK of approximately 4.4 was estimated for the transition between
the most cationic form (35.3 kK) to the form bearing one less proton
(32.2 kK).

Reduction of both 1 and dephosphorylated 1 led to a loss of the low
energy absorbance. Addition of NaBH₄ to 1 at pH 8.5 led to the formation
of an absorption band at approximately 29.9 kK. When the reduction was
done at pH 11, the initial absorption was at approximately 30.9 kK. It
was observed that in order to get reproducible spectral shifts of the
reduction product as a function of pH, it was necessary to first acidify
the reaction mixture after formation of the initial absorption band at
29.9 or 30.9 kK. Reduced 1 exhibited absorption bands at 34.6 kK, pH 3.0;
30.8 kK, pH 8.5; and 32.6 kK, pH 12.0 (Figure 40). These spectral shifts
with pH parallel those for a pyridoxamine derivative (Metzler *et al.*, 1973).
Although a pyridoxamine derivative is indicated, the relative
band heights differ from those for PMP or PM (Peterson and Sober, 1954).
Figure 38. Reaction of 1 with alkaline phosphatase at pH 8.0
Figure 39. Alkaline phosphatase treated at pH 2.5 (A); 5.6 (B) and 11.1 (C)
Figure 40. Reduced at pH 3.0 (A), 8.5 (B), and 12.0 (C)
The shoulder on the low energy side of the spectral bands at pH 8.5 and 12.0 suggested the presence of another species which may account for the differences in the relative intensities of the ionic forms of 1.

The reduced dephosphorylated 1 exhibited absorbance bands at 35.2 kK, pH 2.2; 32.0, pH 8.3; and 33.6 kK, pH 12.0 (Figure 41). Loss of the low energy absorbance for both of these compounds is consistent with the idea that 1 is a Schiff base derivative.

Neither reduction product reacted with ninhydrin. In addition reduced dephosphorylated 1 did not react with alkaline nitroprusside/acetaldehyde to give the characteristic color indicative of a secondary amine (Fiegl, 1966), whereas the control compounds, piperidine and 3 methyl-5,6, 7,8-tetrahydro-2,6-naphthyridin-4-ol (shown below) (Fisher and Metzler, 1969), readily reacted.

![Chemical structure](image)

Alkaline hydrolysis of 1 (refluxed 4-6 hrs, pH 12-13) resulted in the formation of a compound that had electrophoretic and spectral properties similar to those of PLP (Figures 37 and 42, Table 6). Addition of an excess of the hydrolytic product of 1 (concentration based on the
Figure 41. Reduced dephosphorylated at pH 2.2 (A), 8.3 (B) and 12.0 (C)
Figure 42. Alkaline hydrolysis product of \[ \text{1} \] at pH 1.7 (A), 7.3 (B), and 9.5 (C)
extinction of PLP at 25.8 kK) to apotransaminase led to 68% reactivation
of the enzyme when compared to the reconstitution of the apoenzyme with an
excess of PLP. The presence of impurities in the material derived from
the hydrolysis of 1 that bind to the apoenzyme but are inactive could
account for the difference in ability to reactivate. Of the analogs that
have been bound to apotransaminase only a few have the ability to reacti-
vate the apoenzyme to any great extent.

The stability and lack of hypsochromic shift at high pH suggested
as a partial structure for 1 is shown in Figure 43.

Although a Schiff base of this type has not been previously prepared,
two closely related analogs have been synthesized (Figure 43). Both are
stable at high pH. A comparison of the spectral properties of A, B, and 1
are given in Table 7. As can be seen, both B and 1 do not show the hypso-
chromic shift at high pH. Additionally both lack the absorbance band at
27.0 kK for the HP forms. The HP form of A is composed of nearly equal
amounts of two tautomeric forms, HP-(1) and HP-(2) (Figure 44), in which
either the ring nitrogen or the imino nitrogen is protonated (Fisher and
Metzler, 1969). Since both B and 1 have a substituent on the imino nitro-
gen formation of the HP-(2) tautomer or the anionic (P) form at higher pH
is not possible.

In order to account for the differences in pK values (see Table 7)
between 1 and the model compounds, it is first necessary to rationalize
the pK values of the model compounds and other B6 derivatives.

In general there appears to be a correlation between the pK of the
pyridinium nitrogen and the electron withdrawing ability of the 4
Figure 43. Model compounds A and B; partial structure for 1

Table 7. Comparison of spectral properties of 1 with model compounds

<table>
<thead>
<tr>
<th></th>
<th>$H_3P$</th>
<th>pK</th>
<th>$H_2P$</th>
<th>pK</th>
<th>HP</th>
<th>pK</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(^a)</td>
<td>27.7;34.2</td>
<td>1.35</td>
<td>23.3;33.7</td>
<td>6.3</td>
<td>23.2;27.0;34.1</td>
<td>8.86</td>
<td>27.4</td>
</tr>
<tr>
<td>B(^b)</td>
<td>27.7;34.5</td>
<td>0.50</td>
<td>23.0;32.2</td>
<td>6.0</td>
<td>22.8;33.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>30.8(s);35.4</td>
<td>3.90</td>
<td>24.2;34.6</td>
<td>8.7</td>
<td>23.5;33.8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)From Fisher and Metzler (1969).

\(^b\)From Miles et al. (1972).
Figure 44. Tautomers of HP form of A
substituent of the pyridine ring as measured by $\sigma^*$ (inductive effect primarily) or $\sigma_p$ (inductive and resonance effects) (Table 8). The correlation seems to hold for the pK of the phenolic group although steric interactions may complicate the picture. In addition the phenolic pK of the pyridoxamine and Schiff base derivatives may be lower than anticipated as internal hydrogen bonding between the phenolate anion and the protonated amino or imino function would tend to increase the acidity of the phenolic group. Likewise the pK of the amino or imino proton will be greater than expected for these compounds. For both pyridoxamine and the PL-valine Schiff base this pK is approximately 10.5, whereas for A in which internal hydrogen bonding is not possible the pK is 8.9. However the pK of the phenolic group for both compounds A and B is lower than expected which illustrates the influence of the positive charge of the imino nitrogen on the pK of the phenolic group.

It is suggested that partial neutralization of the charge of the imino nitrogen of I by the phosphate group may in part account for the increased pK's of I when compared to compounds A and B. This is consistent with the electrophoretic data which indicated an apparent "loss" of pK$_2$ of the phosphate. Since I served as a substrate for alkaline phosphatase this eliminated the possibility of a phosphodiester.

In all likelihood the high energy bands of I represent hydrated and other tautomeric forms. Comparison of the relative intensities of the high and low energy bands of I to those of the model compounds A and B indicated that, in addition to neutralization of the positive charge of the imino nitrogen, hydration of the imino double bond may contribute to
Table 8. Effect of 4 substituent on pK values of B₆ derivatives

<table>
<thead>
<tr>
<th>R^{a}</th>
<th>σ^{*}</th>
<th>pK₁</th>
<th>pK₂</th>
<th>pK₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CH</td>
<td>0.00</td>
<td>5.35</td>
<td>9.73</td>
<td>-</td>
</tr>
<tr>
<td>-CH₂OH</td>
<td>0.56</td>
<td>4.94</td>
<td>8.89</td>
<td>-</td>
</tr>
<tr>
<td>+ -CH₂NH₃</td>
<td>1.90⁻</td>
<td>3.46</td>
<td>8.13</td>
<td>10.40</td>
</tr>
<tr>
<td>-CHO(FLP)</td>
<td>-</td>
<td>3.34</td>
<td>8.03</td>
<td>-</td>
</tr>
<tr>
<td>+ -C=N⁻&lt;R (PL-Val Schiff base)</td>
<td>-</td>
<td>-</td>
<td>5.88</td>
<td>10.49</td>
</tr>
</tbody>
</table>

^{a}Sigma value is for CH₂N(CH₃)₂, σ^{*} values from Taft (1956), pK values from Harris et al. (1976) and Metzler et al. (1973).
the observed differences in pK's when compared to the model compounds. Since the pK's measured for \( \text{I} \) are macroscopic or stepwise constants for proton dissociation and are related to the microscopic dissociation constants and the equilibrium constants for hydration, the effect of hydration will be to raise the apparent pK values. The microscopic pK's of the hydrated forms are greater than those of the nonhydrated species.

The hypsochromic shift of the low energy bands of \( \text{I} \) relative to those of \( \text{A} \) and \( \text{B} \) (Table 7) is also consistent with a decreased electron accepting ability of the 4 substituent.

Removal of the phosphate apparently increases the extent of hydration as is evident by the decrease in the intensity of the low energy band upon reaction with phosphatase. The band positions of dephosphorylated \( \text{I} \) compare with those of aldehyde hydrates (Harris et al., 1976) although they are slightly hypsochromically shifted. The high energy bands of \( \text{A} \) and \( \text{B} \) are also hypsochromically shifted when compared to the aldehyde hydrates. As would be expected, reduction of dephosphorylated \( \text{I} \) had a small effect on the spectrum.

The hypsochromic shift of the hydrate bands of \( \text{I} \), dephosphorylated \( \text{I} \), \( \text{A} \) and \( \text{B} \) relative to those of the aldehyde hydrates may in part be due to the fact that the 4' carbon of the aldehyde hydrate is dihydroxy substituted, whereas the Schiff base hydrates are carbinolamine derivatives.

It might be expected that the effect of substituting \( -\text{NHCH}_2^- \) (\( \sigma_p = -0.66 \), \( \sigma_m = 0.16 \) for \( \text{NH}_2 \); \( \sigma_p = -0.84 \) for \( -\text{NHCH}_3 \)) for \( -\text{OH} \) (\( \sigma_p = -0.37 \), \( \sigma_m = 0.121 \)) (sigma values from McDaniel and Brown, 1958) would be to decrease the electron accepting ability of the 4 substituent which would lead to a
hypsochromic shift. [An exact correlation is not being attempted. The sigma values are given to indicate what effect the substituent on the 4' carbon may have on the electronic properties of the 4 substituent of the pyridine ring. For example, a hypsochromic shift is observed when a 4' hydroxyl group is replaced with a proton (Figure 45).]

Another factor that may be important in accounting for the differences in the hydrate band positions particularly for the differences between 1 and the model compounds is the effect of the five membered ring. It appears that the effect of a ring is to shift the spectrum hypsochromically as can be seen in Figure 45. It would also appear that the effect of substituting a five membered ring for a six membered ring is to shift the spectrum hypsochromically. For example, the cationic hydrate form of A absorbs at 34.2 kK (Table 7), whereas the same ionic form of pyridoxal hemiacetal absorbs at 34.7 kK.

Analysis of the spectra of 1 and its derivatives and comparison with spectra of other B₆ derivatives support the partial structure shown in Figure 43.

The pmr of 1 is shown in Figure 46. Correlation of the chemical shifts for 1 with those of other B₆ derivatives and Schiff bases (Abbott and Martell, 1970; Korytynk and Singh, 1963) indicate that the peak at δ 2.69 represents the 2-CH₃ group and that at 8.3 is due to the 6-H. The doublet at δ 2.51 arises from the methylene bearing the phosphate. The peaks at δ 7.7-7.9 may be due to impurity or represent the hydrated form. The pmr is consistent with the structure which follows.
Figure 45. Effect of 4 substituent on band position (Metzler et al., 1973)
Figure 46. Proton NMR of \( \text{I} \) in \( \text{D}_2\text{O} \)
The lack of absorption peaks for the 4', 5', and α carbons may, in part, be due to deuterium exchange. In order to keep the height of the HDO peak small, \( \text{\textsubscript{1}} \) was lyophilized 4-6 times from \( \text{D}_2\text{O} \), pD 3.5-4.5

Additionally the methylene protons in the five membered ring may be nonequivalent due to ring puckering which would lead to a broadening of the signal for these protons. This effect is seen in the pmr of pyridoxal hemiacetal (Korytnyk and Singh, 1963; Abbott and Martell, 1970). The methylene protons at the 5' position appear as an AB pattern and in addition one of the protons is coupled to that at the 4' position. Broadening of the 7 methylene protons and the aldimine proton was also observed for \( \text{\textsubscript{A}} \) (Fisher and Metzler, 1969). By analogy, interaction between the aldimine proton and the 5' methylene protons of \( \text{\textsubscript{1}} \) might be expected as well as interaction between the α proton, the aldimine proton and the 5' methylene protons. The effect of these interactions, as well as deuterium exchange, could account for the lack of signals for these protons.
Attempted synthesis of dephosphorylated 1

In an effort to synthesize the dephosphorylated derivative of 1, the pyridoxine compound (II) shown in Figure 47 was synthesized.

To 340 mg (1.64 x 10^{-3} moles) of isopropylidineisopyridoxal (I) was added 100 mg (1.64 x 10^{-3} moles) of freshly distilled ethanolamine and 25 ml of dry benzene. This was refluxed for 4 hours using a Dean-Stark trap. Evaporation under reduced pressure yielded a yellow oil. The residue was dissolved in 5 ml of absolute ethanol and 65 mg of NaBH₄ was added. The reaction could be monitored by nmr by observing the loss of the aldimine (δ 8.3) and the 6 proton (δ 8.1) of the Schiff base and the appearance of a new signal representing the 6 proton of the reduction product (δ 7.85). After 12 hours the reaction was judged complete. The reaction mixture was evaporated under reduced pressure and the residue dissolved in approximately 25 ml of chloroform and extracted three times with H₂O to remove borate. Loss of the product occurs since it is somewhat soluble in water. The chloroform layer was evaporated under reduced pressure and the residue dissolved in 5 ml of 0.1 N HCl and placed in a boiling water bath of 30 minutes. Evaporation under reduced pressure led to the formation of white crystals which could be recrystallized from anhydrous ethanol and ether. Yield was 180 mg or 38% of the theoretical.

High voltage electrophoresis (0.1 M acetate, pH 4.6, 2000 V for 20 min) showed the presence of one compound that migrated 7 cm toward the cathode. The compound was Gibbs positive (Gibbs, 1927) and reacted with nitroprusside/acetaldehyde (Fieg1, 1966) which is indicative of a secondary amine.
Figure 47. Attempted synthesis of dephosphorylated I.
Proton nmr ($D_2O$, pD 9.4, DSS internal standard) showed peaks at $\delta$
2.37 ($s, 3, 2-CH_3$), $\delta$ 2.9 ($t, 2, \text{splitting} = 6 \text{ Hz, } \alpha-CH_2$), $\delta$ 3.7 ($t, 2,
\text{splitting} = 6 \text{ Hz, } \beta-CH_2$), $\delta$ 3.90 ($s, 2, 5'-CH_2$), $\delta$ 4.80 ($s, 2, 4'-CH_2$),
$\delta$ 7.55 ($s, 1, 6-H$). The assignments are based on correlation with spectra
of other B6 compounds. Additionally at pD 2.2 the signal at $\delta$ 3.9 ($5'-CH_2$)
as well as that at $\delta$ 2.9 ($\alpha-CH_2$) were shifted downfield. Protonation of
the 5' nitrogen would be expected to have the most effect on the chemical
shifts of these protons. At pD 2.2 the peak positions seen were $\delta$ 2.75
($s, 3, 2-CH_3$), $\delta$ $\sim$3.4 ($\text{multiplet, 2}$), $\delta$ $\sim$3.9 ($\text{multiplet, 2}$), $\delta$ 4.60 ($s, 2,
5'-CH_2$?), $\delta$ 4.77 ($s, 2, 4'-CH_2$?), $\delta$ 8.43 ($s, 1, 6-H$). The increased
splitting of the $\alpha$ and $\beta$ methylene signals at lower pD indicated that the
protons on the methylene groups were not equivalent, suggesting internal
hydrogen bonding (Figure 48).

The mass spectrum of II indicated peaks at m/e 212.11605 ($M^+$, 7%),
181 (63%), 163 (60%), 152 (100%). The exact mass measured was that ex­
pected for II. The fragment at m/e represents the loss of $\cdot\text{CH}_2\text{OH}$ from the
molecular ion by alpha cleavage (McLafferty, 1973), yielding an even
electron ion. Formation of the fragment with mass 152 may arise from loss
of $-\text{NH=CH}_2$ from the 181 mass fragment perhaps due to displacement by the
4' oxygen yielding a five membered ring.

Oxidation of II was attempted using MnO$_2$. To 5 mg of II was added
5 mg of solid MnO$_2$ in 25 ml of water. The reaction was stirred for 30
minutes at room temperature. After that time the MnO$_2$ was filtered off.
Upon making the reaction mixture alkaline (pH 11), the solution slowly
turned yellow in color and a brown precipitate formed which was filtered
Figure 48. NMR of dephosphorylated \text{1}
off. A spectrum of the material showed a broad absorption band at 23-24 kK which may be the cyclic Schiff base. However with time the low energy band decreased in intensity and shifted to higher energies. This may have been due to further reaction with MnO. Although it was encouraging to see the formation of the low energy absorption band, further work needs to be done on the oxidation. Problems with the oxidation of these types of pyridoxine derivatives have been encountered previously (Brooks, 1961). The mechanism proposed for the inactivation of GAD by serine sulfate is shown in Figure 49.
Figure 49. Proposed mechanism of GAD inactivation by L-serine-O-sulfate
DISCUSSION

Inactivation of GAD by L-Serine-O-Sulfate

The structure proposed for \_1 has interesting implications concerning the mechanism of the decarboxylase and perhaps for other PLP requiring enzymes. The mechanism proposed for the inactivation of GAD (Figure 49) by serine sulfate requires that the Schiff base of the coenzyme and inhibitor have the syn-transoid conformation at some time during the reaction. This is in contrast to the usually accepted syn-cisoid conformation. Since the metal chelates of Schiff bases have a planar syn-cisoid conformation (Figure 50), it was naturally accepted that the Schiff base of

![Figure 50. Schiff base metal chelate](image)

the coenzyme and substrate when bound to the protein also has this conformation.

However Fisher and Metzler (1969) have shown that the spectral properties of a Schiff base with the syn-transoid conformation are very similar to those of aspartate aminotransferase suggesting that this conformation should be considered for PLP containing enzymes. Additionally the
conformational analysis done by Tumanyan et al. (1974) on aldimines of PLP and PLP analogs indicated that the conformations of the 5 substituent were the same for either the syn-cisoid or the syn-transoid conformation with the only difference being in the height of the energy barriers between conformations. Finally the reaction of α⁵-pyridoxal methyl chloride with the B protein of tryptophan synthetase (Miles, 1972) requires that the analog be bound to the protein with the syn-transoid conformation.

Are there any chemical advantages to having the syn-transoid conformation? One possible advantage is that the positive charge on the imino nitrogen will not be decreased due to hydrogen bonding with the phenolate anion as is possible with a syn-cisoid conformation. This would have the effect of increasing the electrophilicity of the 4' carbon which would facilitate the transimination steps that must occur during the enzymatic reactions.

Although the primary evidence for the postulated mechanism is the structure of I, the mechanism accounts for the covalent modification of the protein and the properties of the inactivated enzyme. Most of the steps in the proposed mechanism have their counterparts in mechanisms proposed for other B₆ enzymes. Thus formation of I is analogous to those enzymes catalyzing β eliminations (Davis and Metzler, 1972) with the exception that ketimine formation results from decarboxylation rather than from labilization of the α proton. Formation of IV from III is analogous to the mechanism proposed for the inactivation of aspartate aminotransferase by pyridoxal sulfate (Yang et al., 1974) and trans-5-nor-5-carboxyvinylpyridoxal (Miura and Metzler, 1976). Formation of V from II is
similar to that proposed for the inactivation of the B protein of tryptophan synthetase by \( \alpha^5 \)-pyridoxal methyl chloride (Miles, 1972) except in this instance a five membered ring is formed rather than a six membered ring. Finally formation of \( I \) resembles the quinone methide formation postulated as an intermediate during the condensation of pyridoxine with various nucleophilic compounds (Frater-Schroder and Mahrer-Busato, 1975).

Perhaps the most unusual aspect of the proposed mechanism is the migration of the phosphate group. Although as pointed out in the Introduction, reaction of GAD with the cycloglutamate analogs or reduction of the glutamate–enzyme complex led to dephosphorylation of the coenzyme. Also of interest is the phosphorylation of a threonine residue of apotransaminase during the reaction of N-pyridoxyl-5'-phospho-L-glutamate (Khomutov et al., 1969), indicating that the phosphomonoester linkage can be labilized.

The identity of the amino acid residue that is modified is uncertain. Possibilities are the lysine group to which the coenzyme is normally bonded or the cysteine which is postulated to be near the active site (O'Leary and Brummund, 1974; Fonda, 1976).

The absence of a lag in the rate of inactivation suggests that the enzyme is inactivated by a reactive intermediate generated during catalysis that does not dissociate from the protein.

The curvature of the first order plots when the reaction is done in the absence of chloride may indicate either that the PLP groups within the enzyme differ in reactivity or that a shift in the equilibrium between two protein conformations may have resulted from the modification of a PLP.
residue making further inactivation more difficult. Additionally the existence of different polymeric forms of the enzyme may account for the curvature. These arguments have been used to explain the curvature in the data observed during reconstitution of apoGAD with PLP (O'Leary and Malik, 1972) and the inactivation of cystathionase by substituted hydroxylamines (Beeler and Churchich, 1976).

The effect of chloride on the linearity of the reaction kinetics merits further study especially in view of the fact that chloride has been demonstrated to activate GAD through a cooperative allosteric mode. In addition chloride increases the rate of reconstitution of the apoenzyme with PLP (Huntley, 1970; O'Leary and Malik, 1972; Fonda, 1975) and inhibits the binding of glutarate (Fonda, 1972b).

Comparison of the $k_{obs}$ for the inactivation of the enzyme in 0.05 M acetate ($0.029 \text{ min}^{-1}$) to that for the reaction in pyridine HCl ($0.017 \text{ min}^{-1}$, calculated from equation 4) suggests that acetate accelerates the reaction even though acetate is a competitive inhibitor ($K_i = 20 \text{ mM}$, Fonda, 1972b). This may be similar to the effect formate has on the inactivation of aspartate aminotransferase by $\beta$-chloroalanine (Morino et al., 1974). The rate acceleration by formate was postulated to be due to the binding of formate to the distal carboxyl binding site, thus allowing the active site of the transaminase to be occupied by the inhibitor in a manner analogous to that of the substrate. Such a scheme is attractive (Figure 51) since it would more easily allow the sulfate group to assume a conformation favorable for elimination. Furthermore if the distal anionic group of the substrate (inhibitor) must be protonated upon binding to the
enzyme as proposed (Fonda, 1972a), serine sulfate might not be expected to bind due to the low pK of the sulfate anion. If acetic acid is bound at the distal carboxyl binding site this might explain why serine phosphate could react with the enzyme in a manner apparently similar to serine sulfate, while in pyridine HCl serine phosphate does not inhibit the enzyme (Fonda, 1972a). Further study of the rate of inactivation at different acetate and serine sulfate concentrations may bear out these ideas.

Interaction of the 5' Analogs of PLP with GAD

None of the analogs tested in this study gave any measurable activity upon reconstitution with the apoenzyme. In general this has been true for many of the analogs previously tested with apoGAD. It is not clear whether this lack of reactivation is due to a decreased ability of the substrate to bind to the apo-analog complex or whether the modification has rendered the analog catalytically unreactive even though the substrate is bound.
Decreased ability to bind substrate may be due to failure of the analog to hold the conformation of the protein required for substrate binding or it may in some way sterically interfere with the glutamate binding site. Additionally if phosphate plays a role in transferring a proton from the amino group of the substrate to the γ carboxyl upon binding then the acid-base properties of the 5' substituent must be considered. Furthermore if rotation about an axis connecting the 2-methyl and 5-methylene groups occurs during catalysis as proposed for the transaminases (Ivanov and Karpeisky, 1969), the conformational mobility of the 5 substituent may be important. Elucidation of these factors is difficult particularly for those apo-analog complexes which are inactive. Some parameter other than activity is needed in order to quantitate and compare the effects of the analog relative to the PLP.

One approach that has been demonstrated in this study is the use of the reaction between hydroxylamine and apoGAD-I to study the interaction of glutamate with the complex. The inhibition of oxime formation by glutamate indicated that the interaction of glutamate with the apoGAD-I complex ($K_i = 45-50$ mM) was greatly decreased as compared to the holoenzyme ($K_M = 0.88$ mM). Several factors may be responsible for this decreased interaction. The analog may not be able to protonate the γ carboxyl of the substrate upon binding due to either a lack of proper conformation or suitable acid-base properties. In addition to the difference in the number of negative charges when compared to PLP, the introduction of conformational restraints in the 5 substituent may have precluded formation of the geometry of the active site necessary for substrate binding.
Comparison of the band positions of the apoGAD-I complex (23.0 kK) and the apoGAD-II complex (23.8 kK) suggests that the vinyl function of the 5 substituent of I is conjugated with the ring. This could account for the lower energy absorption of the apoGAD-I complex. This also implies that $\phi = 0^\circ$ since the conformation with $\phi = 180^\circ$ is sterically prohibited due to interaction of the 4' proton with those of the 5 substituent. Since both analogs bind equally well to the apoenzyme (Mekhanik et al., 1972), it appears that one possible conformation for the cofactor when bound to the protein is with $\phi = 0^\circ$, $\psi = 180^\circ$. It would be expected that if these analogs bound in the fully extended conformation they would not sterically interfere with the substrate binding site. The effect of substituting a carboxymethylene group for the phosphate is to increase the dissociation constant of the apo-analog complex by approximately three orders of magnitude when compared to the dissociation constant for the apoGAD-PLP complex (Mekhanik et al., 1972). This difference may be due to the fact that PLP may bind to the apoenzyme fully ionized (Fonda, 1975) which could serve to orient certain amino acid residues of the protein and dictate a specific conformation required for binding of the substrate and catalysis. Thus it is possible that I fails to provide the necessary geometry of the protein required for substrate binding. The extreme specificity of the holoenzyme in binding only certain amino acids (Fonda, 1972a) indicates that the substrate binding site is well defined.

The band positions of the phosphonate analog-apoenzyme complexes (23.4 kK for III; 24.3 kK for IV) again indicated that the unsaturated site of the 5 substituent for the apoGAD-III complex was conjugated to the ring.
This complex readily interacted with glutamate. The position of the absorption band seen immediately after addition of glutamate is similar to that of the high pH form of aspartate aminotransferase (27.6 kK) in which the imino nitrogen is unprotonated and to those complexes for which it is postulated that the imine double bond is not coplanar with the pyridine ring (26.4 kK) (Huntley, 1970; Sastchenko et al., 1971).

The second possibility for explaining the band position is attractive and it is tempting to speculate that upon binding of the substrate, the intramolecular hydrogen bond between the phenolate anion and imino proton is broken allowing rotation about the C₄⁻C₅⁻ bond. This would have the effect of increasing the electrophilicity of the 4' carbon due to increased electron accepting ability of the imino nitrogen and rotation may at the same time orientate the double bond for addition of the substrate. Weakening of the intramolecular hydrogen bond would be aided by having the pyridinium nitrogen protonated. Lack of a suitable base for deprotonation of the amino group of the substrate for addition to the imino double bond may account for the fact that the substrate is not reacted upon further.

The possibility that binding of glutamate leads to dissociation of the imino proton is highly unlikely for the holoenzyme, however it cannot be discounted as an explanation for the initial spectral change seen with the apoGAD-III complex.

The reasons behind the spectral changes that occur after formation of the "26.7 kK intermediate" are not clear. The ΔA/A for the band at 23.6 kK after 20 hours of incubation with glutamate is equal to that of the complex before addition of substrate which would indicate that the Schiff base of the apoenzyme-analog was reformed. However the absorbance that
reappears at 30.2 kK may represent a different species than that which was originally present. The pH dependence of the spectrum of the apoGAD-III complex suggested that higher energy band represented a species analogous to that of the high pH form of the native enzyme that is the 4' tetrahedral adduct. The 30.2 kK band of the apoGAD-III complex was optically inactive, whereas after incubation with glutamate there was a small positive CD band at approximately 330 nm.

Reduction of the initial apoGAD-III complex, the "26.7 kK intermediate" and the complex (complexes?) after approximately 40 hours of incubation with glutamate led to the formation of identical products. The band position at 29.1 kK represents the reduced Schiff base of the apoenzyme-analog complex. The fact that this species absorbs at lower energy than the reduced holoenzyme (29.8 kK) indicated that the vinyl function of the 5 substituent was intact. For comparison reduction of the apoGAD-IV complex yielded a band at 30.4 kK. Furthermore since reduction yielded a single species regardless of the stage of reaction with glutamate suggested that the reaction was reversible.

It is not readily apparent why the apoGAD-IV complex did not react with glutamate in a similar manner. The differences in the band positions of the apoenzyme complexes with III and IV suggest that III is bound to the protein with a conformation in which $\phi = 0^\circ$, $\psi = 180^\circ$, a conformation that could be readily assumed by IV.

It appears that the cofactor can bind with any of the conformations given in Table 1 (see Introduction). Previous study of the binding of 6-methylpyridoxal phosphate demonstrated that this analog is bound tightly
to the apoenzyme \( (K_a = 6.9 \times 10^{-5} \text{ M}) \) (Mekhanik et al., 1972). The catalytic activity was approximately 25-fold lower when compared to the apoenzyme reconstituted with PLP. Since the presence of the 6 methyl group precludes conformations with \( \phi = 0^\circ \) (Tumanyan et al., 1974), the conformation of the analog when bound must be either \( \phi = 120^\circ, \psi = 180^\circ \) or \( 300^\circ \) or \( \phi = 240^\circ, \psi = 60^\circ \) or \( 180^\circ \) (Table 1).

Space filling models show that if addition of substrate were to occur on the si face of the imino double bond and with the orientation shown in Figure 52, the phosphate could transfer a proton from the amino group to

![Figure 52.](image)

the \( \gamma \) carboxyl simultaneously if \( \phi = 240^\circ \) and \( \psi = 60^\circ \). Since the phosphate can freely rotate about \( \psi \) from \( 180^\circ \) to \( 60^\circ \) with \( \phi = 240^\circ \) another possibility exists in that the phosphate could protonate the \( \gamma \) carboxyl (\( \psi = 180^\circ \)) and then rotate about the angle \( \psi \) to deprotonate the \( \alpha \) amino nitrogen (\( \psi = 60^\circ \)). The same considerations apply for addition to the re face except that \( \phi = 120^\circ \) and \( \psi = 180^\circ, 300^\circ \).

The fact that the 6 methyl analog led to partial reactivation of the apoenzyme suggests that one of its possible conformations may be that of
the holoenzyme. In addition these conformations are those which would most easily facilitate phosphate mediated proton transfer if such a transfer does indeed occur. The low catalytic activity may be due to decreased ability of the apoGAD–6 methyl analog to bind glutamate or perhaps since the methyl group is electron donating relative to hydrogen it may have some effect on the electronic properties of the cofactor, particularly on the phenolic group and the pyridinium nitrogen.

The 5'-methylpyridoxal phosphate analog binds very well to the apo-enzyme (K = 1.9 x 10⁻⁶ M) (Mekhanik et al., 1972). The complex has a K_M for glutamate slightly higher than that of the holoenzyme and a V_max four times lower. Since this analog can assume conformations with φ = 0°, does this suggest that another possible reason for the low catalytic activity seen for the 6 methyl analog is due to the fact that φ must equal 0° at some time during catalysis, perhaps for binding of the substrate?

Finally with a conformation of φ = 120° or 240° and ψ = 180°, formation of II (Figure 49) in the reaction of GAD with serine sulfate could readily occur. A space filling model of III indicates that the 4' proton is perpendicular to the pyridine ring and that the 5' methylene group can easily rotate from 240° to 270° for elimination.

Although this is a meager beginning and the discussion of the conformation and role of the phosphate in catalysis speculative, further work in this area should prove fruitful. In particular the inhibition of the reaction between hydroxylamine and the apo–analog complex by glutamate or, in those cases where the apo–analog complex is active, glutarate should be most useful in sorting out the factors that are necessary for activity.
At least one would be in a position to say whether or not the substrate or inhibitor interacts with an apo-analog complex as well as it does with the holoenzyme.
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